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A VIRUS DISEASE OF WESTERN HEMLOCK LOOPER, LAMBDINA FISCELLARIA LUGUBROSA (HULST) (LEPIDOPTERA: GEOMETRIDAE)[1]

S. M. SAGER[2]

Abstract

Some observations have been made on the infectious agent, the histopathology, and the incubation period of a polyhedrosis in western hemlock looper larvae. External and internal symptoms are generally similar to nuclear polyhedrosis in other Lepidoptera. Infection is first detectable in fat body nuclei, other susceptible tissues being blood, hypodermis, and tracheal matrix. Larvae subjected to a short period of starvation just prior to oral infection show a shorter incubation period than non-starved larvae. The polyhedral inclusion bodies range from 0.5 μ to 5.0 μ in diameter. Electron micrographs of dissolved polyhedra show their contents to be largely rod-shaped viruses measuring approximately 40 mμ $\times$ 290 mμ.

Introduction

A collapse of a serious outbreak of western hemlock looper, *Lambdina fiscellaria lugubrosa* (Hulst), on Vancouver Island in 1945–47 was associated with the occurrence of a virus epizootic[3] (3). The disease appeared to be widespread in the Pacific Northwest since a polyhedrosis was also diagnosed in larvae from the State of Washington (5, 6). As in the nuclear polyhedroses of other insect species the disease is characterized by the development of polyhedral inclusion bodies in the nuclei of certain cells. Observations made at the time of the Vancouver Island outbreak indicated that it was an effective disease in field populations of the looper. Since this insect is a major defoliator in western forests, a study of the role of the polyhedrosis as a biological control agent is of particular interest. This paper contains some preliminary observations made by the author, a description of the pathogenic agent, and the histopathology and the incubation period of the disease in laboratory-reared larvae.

[1]Manuscript received May 16, 1957.
Contribution No. 397, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.

[2]Forest Biology Laboratory, Victoria, British Columbia.

[3]Unpublished records by K. Graham, G. R. Wyatt, and M. G. Thomson. Forest Biology Laboratory, Victoria, B.C.

[The previous number of Can. J. Microbiol. (3, 673–798) was issued August 22, 1957.]

Methods

The source of virus inoculum for infection experiments was a small number of polyhedrosis-infected hemlock looper larvae which had been kept in a dried condition for 6 or 7 years. These cadavers were pulverized and fed as a water suspension to laboratory-reared larvae. All dead larvae from such infections were decomposed in water at room temperatures and the resultant suspension of polyhedra partially purified by repeated washing and centrifugation (1, 2). A cream-colored suspension of polyhedra in distilled water was prepared and used as the inoculum for all subsequent infection experiments and observations on the infectious agent.

In the preparation of electron micrographs of the virus particle, a series of trial and error tests showed that in a 1:1 mixture of polyhedral body suspension and 0.01 *M* Na_2CO_3 the polyhedra were dissolved sufficiently in 20–30 minutes to reveal their contents and several stages in the dissolution process. Loopfuls of this alkaline solution were then placed on collodion-covered grids, fixed with osmic acid vapors, and shadowed with uranium. Micrographs were taken at 11,100× on an RCA type EMU-2A electron microscope and optically enlarged to 50,000× for measurements of virus particles.

To determine the incubation period of the disease, we allowed larvae to ingest a large drop of the suspension of polyhedra, after which they were supplied with polyhedra-contaminated foliage for 32 hours. After this, the larvae were fed foliage free of virus. The incubation period was defined as the interval between first exposure to polyhedra and death of the larvae. Effects of a short period of starvation just prior to infection were studied in larvae held for 18–24 hours without food and then fed a drop of the polyhedral body suspension and only uncontaminated foliage. Control larvae were given distilled water or foliage dipped in distilled water in place of the suspension. Larvae were reared in groups of 5 or 10 per container. Diagnosis consisted of dark-field microscope examination of tissue smears.

Histopathology of the disease was followed in a series of larvae examined at 24-hour intervals following infection. Prior to killing and fixing of the larvae, blood smears were made from each specimen and examined with dark-field illumination. The larvae were then fixed in aqueous Bouin's solution. Paraffin sections were made and stained with Heidenhain's iron haematoxylin and eosin.

Results

Under both dark-field illumination and the electron microscope, inclusion bodies isolated from the western hemlock looper appear as bright, highly refractive polyhedra. The number of sides varies but a round-cornered triangular outline is most common (Fig. 1). Some have so many sides that they appear almost round (Fig. 2). The diameter of 50 polyhedra isolated from larvae in various stages of the disease ranged from 0.5 μ to 5.0 μ.

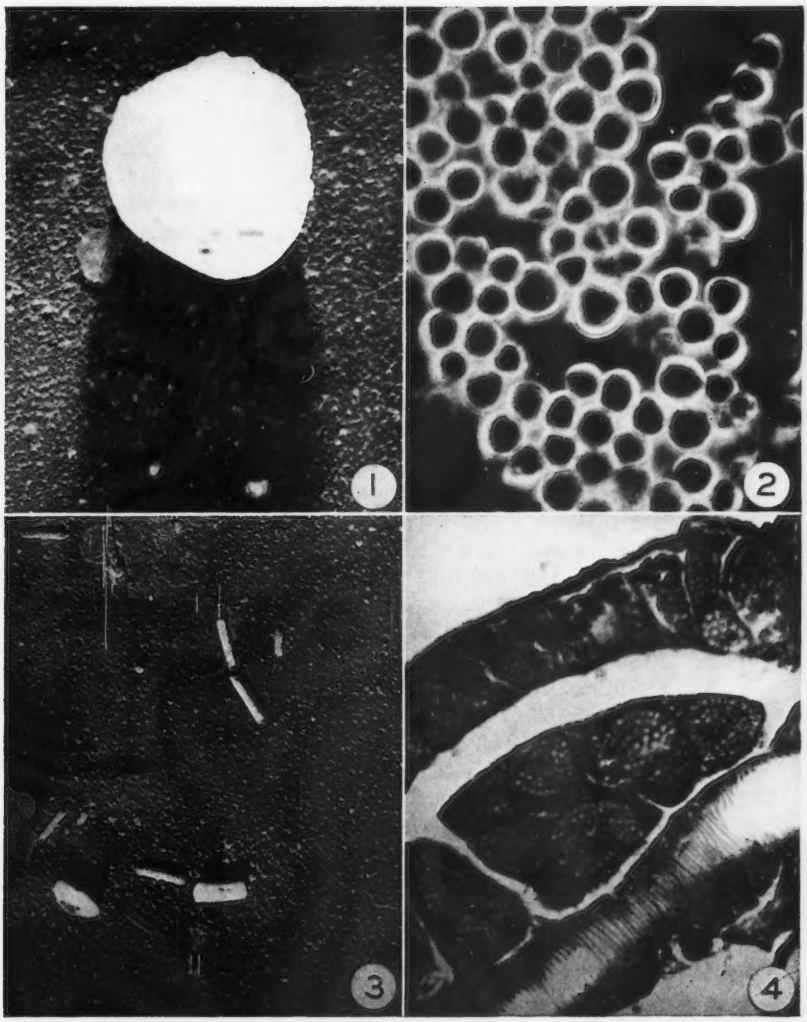

FIG. 1. Polyhedron shadowed with uranium; electron micrograph, approximately 25,000×.
Fig. 2. Polyhedra, dark-field illumination; 1800×.
FIG. 3. Electron micrograph showing intact virus rods or bundles, a membrane being dissolved from a rod and slender virus particles; approximately 25,000×.
FIG. 4. Section showing polyhedra in hypertrophied nuclei of hypodermis, fat, and tracheal matrix; 500×.

Complete dissolution of polyhedra in weak Na_2CO_3 solution was observed with the dark-field microscope. The first indication of dissolution is a gradual fading of the refractive material and appearance of short gaps or breaks in the bright outline. These gaps gradually increase in size, the rate depending on the concentration of alkali. When the refractive material has all but disappeared, very small bluish particles can be seen vibrating within the now swollen polyhedra. The latter finally becomes a round thin-walled structure and may remain in this state for a considerable period of time. If the polyhedron is disturbed or subjected to a higher concentration of alkali the thin wall breaks, releasing the vibrating particles—presumably the virus rods.

Electron micrographs prepared to date have not been for the purpose of detailed morphological studies but are of sufficient quality to reveal that the polyhedra contain principally sausage- or rod-shaped bodies typical of nuclear polyhedrosis (1) (Fig. 3). These rods apparently are surrounded by a thin membrane which dissolves in Na_2CO_3 solution, and contain perhaps two or more infective virus particles (1, 4). The average dimension of 10 of the better specimens of virus particles was 40 mμ × 290 mμ.

External and internal symptoms of the disease are similar to those observed in other Lepidoptera infected with nuclear polyhedrosis (5). Gross symptoms are not obvious until late stages of infection, the first symptom being a reduction in feeding. Larvae that have not been starved prior to infection show a decrease in feeding activity at about the 12th day. In the next stage the larval body becomes slightly lighter in color because of the presence of polyhedra in the hypodermis and fat body. The larvae then soon cease feeding and become inactive and somewhat puffy in appearance. Death usually occurs a day or two after the larvae cease to feed. At death the body is completely limp or wilted and its contents appear to be almost entirely replaced by fluid. One or more of the legs, usually the hind pair, may become firmly attached to the foliage or the side of the rearing container with the rest of the body hanging down limply. The integument is extremely fragile and readily ruptures when touched, releasing an odorless, cream-colored fluid.

On the basis of at least 15 larvae examined at each 24-hour interval after infection the following outline of the progress of larval infection can be given. Symptoms were first detectable on the 6th day in the form of an aggregation of chromatin in fat-body nuclei. Within the next 2 days the nuclei of the hypodermis, blood, and tracheal matrix also showed similar nuclear changes, and in some nuclei small refractive bodies, presumably polyhedra, could be seen. These polyhedra increased in size and number, completely filling the nuclei by the 11th to 13th day (Fig. 4). Just before death of the larvae, polyhedra were found free in the blood fluid.

Table I summarizes the results of infection experiments to determine the incubation period of the disease. Sixty-seven per cent of the larvae which were not starved prior to the experiment died from polyhedrosis infection. The average incubation period in these was 15.5 days, with a range of 13–

19 days. Sixty per cent of the starved group of larvae died and showed an average incubation of only 11 days with a range of 7–15 days. It has been reported (1) that the incubation period is reduced by increasing the amount of virus fed to larvae. The present results show that larvae given a greater exposure to polyhedra, that is both a drop of polyhedra suspension and contaminated foliage, had a longer incubation period than the larvae given only the single drop of suspension. The shorter incubation in the latter group may in some way be related to the short period of starvation to which they were subjected just prior to the experiment.

TABLE I

INCUBATION PERIOD OF A POLYHEDROSIS IN WESTERN HEMLOCK LOOPER, COMPARING NON-STARVED LARVAE TO LARVAE WHICH WERE STARVED JUST PRIOR TO ORAL INFECTION BY POLYHEDROSIS

	Feeding condition prior to infection	Number of larvae exposed to infection	Range in incubation period (days)	Average incubation period (days)	Percentage mortality
Infected larvae	Not starved	150	13–19	15.5	67
	Starved	165	7–15	11.0	60
Uninfected larvae (controls)	Not starved	40	—	—	0
	Starved	30	—	—	0

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THE INFLUENCE OF THE INOCULUM ON VARIABILITY IN COMPARATIVE NUTRITIONAL EXPERIMENTS WITH FUNGI[1]

W. A. TABER

Abstract

This study was designed to determine whether the method of preparing an inoculum could influence the results obtained from comparative nutritional experiments.

The quantity of inoculum used to start cultures of *Claviceps purpurea* clearly affected the rate of growth, and to a lesser extent total growth on mannitol, and growth rate varied directly with increasing inoculum size.

The quantity of inoculum used to start cultures also affected the uniformity of growth among replicas. As the amount of inoculum decreased, the variation among replicas increased and this affected the power of the experiment to distinguish between similar mean growth responses. These observations suggested that standardization of the quantity of inoculum might be profitable. It is proposed that the smallest amount of inoculum represented by a coefficient of variation of about 10% be considered an acceptable inoculum. Such an inoculum is large enough that growth among replicas is not too irregular and small enough that nutrients are not unnecessarily added to the experimental medium through the inoculum.

The effect of heterokaryosis on uniformity of growth is demonstrated with *Isaria cretacea* van Beyma.

Introduction

A comparison of the nutritional data obtained in this laboratory during a study of several strains of *Claviceps purpurea* with those published by other laboratories suggested that the disagreements might be due to the use of different experimental procedures and, in particular, to the use of inocula prepared in different ways (15).

The possible sensitivity of growth to the method in which the inoculum is prepared appears not to be generally appreciated because discussion of the inoculum as it pertains to variation appears only occasionally in the literature. For this reason a study was undertaken to determine whether the method of preparing an inoculum could cause significant variation in the growth response of a fungus to an experimental condition. Variation attributable to techniques is significant because it may affect the interpretation of data within an experiment or the comparison of data obtained from different laboratories. In either event the variation can be reduced by changing the experimental procedures.

A second type of variation results from the differences in growth between cultures intended to be identical (replicas) and it exists because even under the most exacting of methods growth conditions cannot be duplicated perfectly. It may be viewed as the residual variation remaining after attempts have been made to reduce variation by improvement of experimental methods.

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Since this variation cannot be eliminated, it must be taken into consideration during the comparison of mean growth responses. This can be achieved best by statistical analyses of the data. Brief mention of this type of variation is made to render the discussion of variation more complete.

Material and Methods

The experimental procedures used in this study have been described in detail in a previous publication (15). In brief, inocula were prepared by homogenizing shaken cultures and then washing in saline. The mycelia were homogenized again after the final suspension in saline. Growth was measured by weighing the mycelium of a culture on a heat-dried, individually tared filter paper and recorded as the mean and standard error of several cultures. Such a group of cultures is hereafter referred to as a replicate and each culture of a replicate is called a replica. Exceptions to these procedures are noted in the text.

Variation Due to the Inoculum

Effect of the Quantity of Inoculum on Uniformity of Growth

If the variation in growth among replicas is large, the mean will not give an accurate estimate of growth and consequently the comparison of two or more such means may not be accurate. The uniformity of growth among replicas can be influenced by the quantity of inoculum used to start the cultures. This variation decreases sharply up to a limit as the quantity of inoculum increases (Table I, and Fig. 1 in reference 15), and is inversely correlated with the inoculum size, not the amount of growth. When the size of the inoculum is held constant, however, variation (expressed as the coefficient of variation) does decrease with increasing amount of growth.

TABLE I

EVIDENCE THAT THE COEFFICIENT OF VARIATION IS INVERSELY CORRELATED WITH THE QUANTITY OF INOCULUM RATHER THAN THE AMOUNT OF GROWTH

Claviceps purpurea PRL 1555

Inoculum size (ml.)	Mean mg. dry mycelium/flask	Coefficient of variation, %	Days incubated
3.0	181.9	3	3
0.5	241.0	6	6
0.1	194.8	9	8

INOCULUM: 1.1 ml. is equivalent to 0.9 mg. dry mycelium.

This variation cannot be reduced simply by inoculating media with large quantities of mycelia, however, because, as it will be shown, the response of a fungus to a nutrient can be influenced by the size of the inoculum.

There is a problem, then, in the selection of a quantity of inoculum to be used in an experiment. A rational method of selecting an inoculum size would be to determine the smallest amount not causing undue variation. This requires expressing the variation within replicates and setting a limit to the

amount that will be tolerated without necessitating rejection of the data. Variation within a single sample (replicate) can be expressed as the coefficient of variation (*CV*), which is computed from the standard deviation *s* (13):

$$s = \sqrt{\frac{\Sigma_{i=1}^{n} (x_i - \bar{x})^2}{n - 1}}$$

$$CV = \frac{s}{\bar{x}} \times 100$$

where Σ is the sum of the squares of the differences between the size of each replica x and the mean $\bar{x}$, and n is the number of replicas. The *CV* decreases as the variation decreases. Examination of cultures started from vegetative inocula of various fungi reveals that exceptionally uniform growth among replicas is represented by a *CV* ranging from 2 to 6%. Cultures grown on a minimal synthetic medium and started from quantities of vegetative mycelia considered not to be excessive are represented by *CV*'s ranging from 5 to 15%. A *CV* of approximately 10% could be accepted as the limit and an inoculum characterized by a larger value would be considered too small. In practice, the smallest inoculum causing variation equivalent to a *CV* of about 10% using the *control* medium would be acceptable and the unnecessary introduction of nutrients into the experimental medium by a larger inoculum would be avoided.

Claviceps purpurea has been shown previously (15), and will be shown in the section to follow, to utilize mannitol poorly when either an "acceptable" or small inoculum (determined on the control medium of No. III containing glucose (16)) is used, but to grow on mannitol almost as well as on glucose when an "excessive" inoculum is used. No attempt is made here to decide which information is the more meaningful but it does seem reasonable to expect that methods capable of revealing differences provide more information than those incapable of showing differences. The above criterion for an "acceptable" inoculum is based upon this assumption.

Effect of the Quantity of Inoculum on Nutrient Utilization

Some of the published reports do not agree on the relative utilization of mannitol for growth by *Claviceps purpurea* (1, 10, 11, 15, and 17). The four cultures studied in this laboratory under identical conditions, however, responded almost identically with the nutrient variables examined, and this suggested that the lack of agreement in other reports might be due to the use of different procedures rather than to the existence of nutritionally-different strains. Since all of the cultures were not available, one strain, PRL 1555, was examined to determine if it could be made to respond like the other cultures by varying experimental conditions.

It was found that this culture could be made to respond to mannitol in varying degrees (Tables II, III, and IV) by changing the quantity of inoculum, and it thus could be made to resemble the other cultures. Cultures started

with a small quantity of inoculum grew poorly on mannitol, while cultures started from larger inocula grew well on mannitol. Growth increased during a prescribed incubation interval as the quantity of inoculum increased (Table III) and the lag period decreased. While these facts suggested adaptive utilization of mannitol, the fact that cultures started from inocula grown on either glucose or mannitol grew at the same rate on the two sugars (Table IV) is not consistent with this interpretation.

TABLE II

FACTORIAL EXPERIMENT (TWO COMBINED) DESIGNED TO TEST THE EFFECT OF CARBOHYDRATE CONCENTRATION, COMPOSITION, AND INOCULUM SIZE ON GROWTH RATE

Claviceps purpurea PRL 1555

Four days

Mg. dry mycelium/flask culture

Glucose				Mannitol			
50 g./l.		15 g./l.		50 g./l.		15 g./l.	
Inoculum (ml.)				Inoculum (ml.)			
2.0	0.2	2.0	0.2	2.0	0.2	2.0	0.2
264.1	33.1	261.2	116.2	124.1	11.9	86.8	9.4
245.0	78.3	265.2	147.8	243.0	9.7	141.2	8.1
252.6	141.8	235.4	98.1	169.7	11.3	166.9	1.3
259.2	191.8	255.4	58.4	245.6	5.9	172.4	10.8

Source of variation	Degrees freedom	Significance	Source of variation	Degrees freedom	Significance
Replicate	3		Replicate	3	
Treatment	3		Treatment	3	
Glucose	1	No	Mannitol	1	No
Inoculum	1	Yes*	Inoculum	1	Yes*
Interaction	1	No	Interaction	1	No
Interaction error	9		Interaction error	9	
Total	15		Total	15	

*Significant ($P < .05$). Interaction on mannitol almost significant.

Inoculum: 0.2 ml. is equivalent to 0.4 mg. dry mycelium. The inoculum was grown on a medium consisting of 1% glucose and 1% yeast extract in distilled water.

TABLE III

THE EFFECT OF THE QUANTITY OF INOCULUM ON MANNITOL UTILIZATION BY *Claviceps purpurea* PRL 1555*

Seven days

Mg. dry mycelium/flask culture

Quantity of inoculum (ml.)		
0.1	2.0	4.0
9.4 ± 1.7†	84.5 ± 9.5	122.7 ± 10.4

*Four replicas. The inoculum was homogenized and washed mycelium was suspended in saline.

†Mean and standard error.

INOCULUM: 1.0 ml. is equivalent to 0.5 mg. dry mycelium. The inoculum was grown on medium III (16).

TABLE IV

EFFECT OF INOCULUM SIZE AND HISTORY OF INOCULUM ON COMPARATIVE UTILIZATION OF GLUCOSE AND MANNITOL*

Claviceps purpurea PRL 1555

Mg. dry mycelium/flask culture

	Inoculum grown on glucose				Inoculum grown on mannitol			
	Large inoculum (1.0 ml.)		Small inoculum (0.1 ml.)		Large inoculum (1.0 ml.)		Small inoculum (0.1 ml.)	
Days	Glucose medium	Mannitol medium	Glucose medium	Mannitol medium	Glucose medium	Mannitol medium	Glucose medium	Mannitol medium
3	161.7†	28.2± 0.6	9.9± 1.0	3.6± 0.6	159.9± 7.0	27.2± 0.1	14.6± 2.0	3.3± 0.5
6	—	232.6±11.1	214.0± 7.5	76.9± 9.0	250.9± 3.0	240.2± 8.9	167.2±19.5	64.3± 8.0
9	—	313.4± 5.2	217.9±10.1	270.9±16.0	228.7±11.1	261.7±33.5	155.6±12.5	205.0±46.0

*Medium III (16).

†Single culture.

INOCULUM SIZE: (*a*) Grown on glucose; 1.0 ml. is equivalent to 1.2 mg. dry mycelium.
(*b*) Grown on mannitol; 1.0 ml. is equivalent to 0.7 mg. dry mycelium.

Effect of the Inoculum History on Growth Rate

The medium in which the inoculum is grown influences subsequent growth when utilization of a substratum requires adaptation, and also in some instances when adaptation is not involved. The latter is true when one is growing an inoculum on a rich, complex, organic medium and when using it to inoculate a minimal synthetic medium. Jennison *et al.* (8) found the effect of carry-over could be eliminated by three serial transfers through the experimental medium. The difference in growth rate of a culture grown on a complex organic medium and a minimal synthetic medium is shown in Table V. The medium used for growing the inoculum will not always affect utilization of a substratum. Utilization of mannitol and glucose by *Claviceps purpurea* was not affected by the media used in the experiment described in Table IV.

TABLE V

EFFECT OF THE COMPOSITION OF THE MEDIUM USED TO GROW THE INOCULUM ON GROWTH RATE IN SYNTHETIC MEDIUM III

Seven days

Alternaria PRL 1671*

Inoculum medium	Mg. dry mycelium/flask culture	
Glucose–yeast extract No. II (16)	320.3 ± 37.1	$t = 3.1$†
Synthetic medium No. III (16)	203.1 ± 9.0	
Second serial transfer on medium No. III (16)	192.0 ± 13.1	$t = 0.8$

*Does not require added vitamins.
†Significant ($P < .05$).

Effect of Heterokaryosis on Growth Rate

The fungus itself may cause irregularity of growth if it is a heterokaryon composed of components capable of different growth rates. While it is difficult to assess the significance of this variation, the fact that it exists warrants its recognition. This is particularly true now that a heterokaryon has been shown to synthesize an enzyme not present in either homokaryon (5). Jinks (9) demonstrated a difference in growth rates of homokaryons isolated from natural heterokaryons of *Penicillium*. An example of this type of variation is given below.

A natural heterokaryon of *Isaria cretacea* van Beyma PRL 1452 consists of two homokaryons (A and B) growing at different rates on a minimal synthetic medium (Table VI and Fig. 1) but not on a complex organic medium. These homokaryons are distinguishable morphologically by the presence of massive sterile coremia on certain media in component A and the complete absence of coremia in component B, and by the presence of sectors of component B in heterokaryotic colonies grown on agar media not favoring coremia formation. The heterokaryon possesses the greatest variation within replicates when synthetic media are inoculated with dilute suspensions of spores of each (Table VI). Complete separation of the homokaryons into different flasks within a replicate could also result in variation. Pontecorvo (12) discusses various nuclear changes.

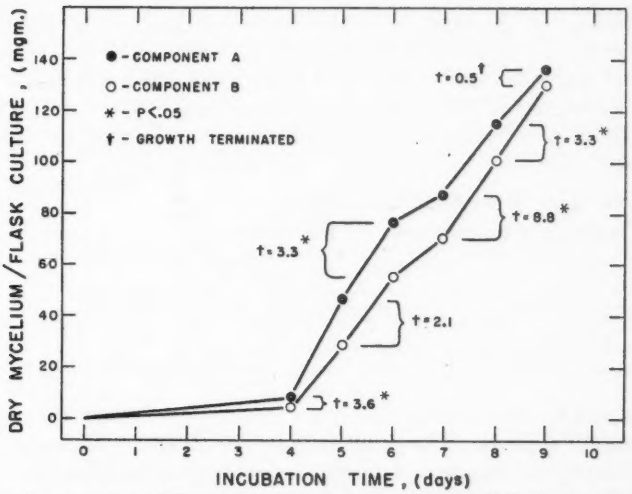

FIG. 1. A comparison of the growth rate on medium No. III (16) of components A and B of *Isaria cretacea*. The inoculum of each was 0.1 ml. filtered and washed spores having a Klett reading of 15. The count of five measurements on each using a haemocytometer was: A (373 ± 18) × 10^3 spores and B (353 ± 10) × 10^3 spores.

TABLE VI

A COMPARISON OF GROWTH AND VARIATION OF COMPONENTS A AND B AND THE PARENT HETEROKARYON P OF *Isaria cretacea* WHEN GROWN ON MEDIUM No. III (16)*

Seven days

Measurements	Component A	Component B	Parent P
Mg. dry mycelium plus spores collected on No. 2 filter paper	131.8 ± 2.1	98.9 ± 5.0	110.9 ± 7.5
Coefficient of variation	3.8%	5.0%	7.5%
Mg. dried spores in filtrate collected by centrifugation	3.1	0.9	Not determined
Klett-Summerson turbidity readings of filtrate	21	10	15
Remarks	Growth as spores from phialides, hyphal wefts, and some pellets. No separation of supernatant on standing	Growth as pellets with some spores. Clear supernatant on standing	Some flasks resemble more closely one component, others the remaining component

Six replicas.

INOCULUM: 0.1 ml. of filtered and washed spores adjusted to colorimeter reading of 15 using red filter No. 64. Spore count per 0.1 ml. estimated by haemocytometer count of five samples was: Component A (490 ± 16) × 10^4; component B (578 ± 22) × 10^3; and P (249 ± 20) × 10^3.

Variation Inherent in the Experiment

Regardless of the techniques employed there will always be some variation within replicates and this cannot be ignored in the comparison of means of similar magnitude. This second type of variation is by definition the uncontrollable variation of a given experiment and it thus requires consideration other than physical manipulation of the experiment. This is achieved by the

proper design of an experiment and statistical analysis of the data. See references 3, 6, 7, 13, and 14 for appropriate discussions. While this subject cannot be discussed here, reference is made to two analyses which are particularly appropriate for analysis of nutritional data. They are the t test and the analysis of variance. Their usefulness is illustrated in the examples to follow.

Comparing Two Mean Growth Responses

Two means can be compared by the t test:

$$t = \frac{\bar{x}' - \bar{x}''}{\sqrt{\frac{\sum_{i=1}^{n_1} (x'_i - \bar{x}')^2 + \sum_{j=1}^{n_2} (x''_j - \bar{x}'')^2}{n_1 + n_2 - 2}} \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where $\bar{x}'$ and $\bar{x}''$ are the two means and x'_i and x''_j the respective measurements of the sets of replicas. The significance of the difference between the means, considering their respective internal variation, is determined by entering a tabulated t table at $(n_1 + n_2 - 2)$ degrees of freedom and observing the probability of a difference of this size or greater occurring by pure chance. As a rule, a probability $P > .05$ is taken to indicate that a significant difference, i.e. one not due merely to chance, was not demonstrated. The alternative to such an analysis when comparing similar values is to compare the means from personal impression, and this may introduce bias. The data of Fig. 1 have been analyzed by the t test in order to compare growth rates of the two components during various stages of growth.

Comparing Several Means

The most convenient design allows for a direct comparison of several means, for instance, the comparison of the growth of a fungus on several carbon sources. The analysis of variance is used, which serves the same purpose as a combination of t tests but is more comprehensive, because the variation is pooled. An example is given in Table VII. See references 2 and 4 for a discussion of this analysis. If P, estimated from the analysis, indicates there is a significant difference among means, the next step is to determine which mean(s) is different. Snedecor (13) has recently modified Tukey's test to give a ready method of isolating the different mean(s). It is the D test, and is carried out as follows, using the data of Table VII.

1. Standard error $= \sqrt{\frac{\text{interaction error}}{\text{number of replicas}}} = 48.$
2. Q is read from Snedecor's table using 5 as the number of treatments and 8 as the degrees of freedom. $Q = 4.9$.
3. $D = se \times Q = 236$.
4. The number of possible comparisons is (number of means $\times$ number of means $- 1)/2 = 10$.
5. The comparisons are made as shown in Table VII.

The pairs of means represented by a value exceeding 236 differ significantly.

Even if it is felt that one could distinguish between similar means by eye in a given instance, there is a distinct advantage in being able to refer to the comparison by a test and a value that is meaningful to every reader. As long as statistical analyses are used as aids to judgment and are not considered substitutes for careful procedures or for the necessity for repeating experiments, this advantage is real.

TABLE VII

EFFECT OF INOCULUM SIZE ON PRODUCTION OF ERGOT ALKALOIDS BY *Claviceps purpurea* PRL 1555

Sixty days*

μg. total alkaloid/liter culture

Data

	Inoculum (ml.)					
Replica	0.1	0.5	1.0	5.0	10.0	Total
1	708	1176	934	954	754	4526
2	856	1242	898	860	820	4676
3	782	1078	1098	946	744	4648
Total	2346	3496	2930	2760	2318	13850
Mean	782	1165	977	920	773	

Analysis of variance

Source of variation	Degrees freedom	Sum of squares	Mean square	*F*
Total	14	368,430	26,316.43	
Inoculum	4	312,286	78,071.50	11.7†
Replica	2	2,545	1,272.50	
Interaction error	8	53,599	6,699.87	

Isolation of different means by *D* test‡

Inoculum	$\bar{x}$	$\bar{x}-773$	$\bar{x}-782$	$\bar{x}-920$	$\bar{x}-977$
0.5 ml.	1165	392	383	245	188
1.0 ml.	977	204	195	57	—
5.0 ml.	920	147	138	—	—
0.1 ml.	782	9	—	—	—
10.0 ml.	773	—	—	—	—

*Medium No. I (16).
†Significant ($P < .05$).
‡Pairs of means represented by a value larger than 236 are different.
INOCULUM: 1.0 ml. was equivalent to 2.2 mg. dry mycelium.

Acknowledgment

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PRODUCTION OF AERIAL MYCELIUM AND UREDOSPORES BY MELAMPSORA LINI (PERS.) LÉV. ON FLAX LEAVES IN TISSUE CULTURE[1]

F. L. M. Turel and G. A. Ledingham

Abstract

Dense, felt-like growth of aerial mycelium of *Melampsora lini* (Pers.) Lév. was obtained when surface-sterilized, rust-infected cotyledons of flax were put on a modified Knop medium, containing fresh, ripe coconut milk, sucrose, and Difco Bacto-Agar. The mycelium remained fully dependent on the living host tissue, but could easily be collected free from host material in quantities sufficient for respiration and small scale chemical studies. Slight modification of the method allowed production of appreciable quantities of uredospores free from contaminating microorganisms.

Introduction

Gautheret (4) and White (13) developed a technique for the cultivation of plant tissues in artificial media. The possibility of using this technique for the cultivation *in vitro* of obligate plant parasites, free of contamination, on their respective host tissues was very attractive. Morel (8), however, was not successful in infecting the healthy callus tissue with uredospores or basidiospores of the appropriate rust, but did suggest the use of surface-sterilized, rust-infected tissue for future experiments. Following this suggestion, Hotson and Cutter (7) obtained uncontaminated cultures of juniper gall tissues infected with *Gymnosporangium juniperi-virginianae* and, eventually, independent growth of the rust fungus on agar media.

Our attempts to repeat the work of Hotson and Cutter (7) have been unsuccessful to date. About 1000 cultures of infected tissue of juniper galls from different parts of the country were grown and in none has there been saprophytic growth of the rust mycelium on the agar medium. Tissue cultures of rust-infected leaf fragments of sunflower, safflower, and flax plants were also developed on both liquid and solid media without obtaining growth of rust mycelium apart from the leaf. However, mycelial growth of *Melampsora lini* on flax leaves could be stimulated to such a degree by certain culture methods that hyphae, of about 1 to 2 mm. length, formed a thick mycelial mat over the leaf epidermis. The technique used in these experiments with flax rust and the results with different media are described in this paper.

Materials and Methods

Uredospores of *Melampsora lini* had been collected originally in the field, but the rust was propagated for many generations on greenhouse flax plants before the tissue culture experiments were started. The flax varieties "Bison"

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and "Redwing" were used and proved equally suitable. The powdery spores were shaken off the plants and stored at 1° C. in gelatin capsules, if necessary for several months. In general, germination varied from 60% in freshly collected material to 30% in spores stored for 4–6 months.

Media

Knop's medium, as modified by Gautheret (4, p. 52, Medium I), and Gautheret's medium (4, p. 51, footnote), without ferric chloride but with Berthelot's solution (4, p. 52, footnote), were most frequently used for tissue culture. However, Heller's medium (5, 12) and White's medium (13, p. 103) were also tested. Unless mentioned otherwise, 2% sucrose, 10% coconut milk, 10.0 mg./liter cysteine hydrochloride, 1.0 mg./liter thiamine hydrochloride, and 0.1 mg./liter indole-3-acetic acid were added to each medium. The pH of the media on preparation varied from 4.5 to 5.6 . For solid media, 0.8–1.0% agar was added.

Growth Conditions

The flax seedlings were grown in pots in the greenhouse. The plants were inoculated before the full development of the cotyledons, just when the first pair of secondary leaves appeared, by spraying them with water and then dusting them with dry uredospores. The pots with the inoculated seedlings were placed in a moist chamber for 18–24 hours after inoculation, and then returned to the greenhouse where light intensity was kept between 600 and 900 ft-c. Intensities over 1000 ft-c. hindered the development of rust pustules (1). The first sign of rust infection was the appearance of light green spots on the leaves after 5 to 8 days.

The most heavily infected cotyledons were removed from the plant at an early stage of rust development shortly before the uredospore pustules broke open. They were cut in half if mycelial growth was desired and left intact for spore formation. The pieces were sterilized twice in 4% Javex (NaClO with 7% available chlorine) and a few drops of a wetting agent, the first time for 10 minutes and the second time for 4 minutes in fresh solution. Then they were rinsed in sterile water and placed on the media in test tubes, with abaxial side on the medium if mycelial growth was to be favored, or with adaxial side on the medium if spores were to be collected. Sixty to ninety per cent of these cultures were free from contaminants. The cultures were grown at a temperature of 20 to 23° C. under constant bluish-white fluorescent lighting (300–600 ft-c.).

Results

Description of the Growth of the Rust and the Host

The appearance of the rusted tissue cultures was the same on solid as on liquid media. The first aerial growth of rust mycelium occurred on leaves, cut in half and floating on liquid Gautheret-medium, after 4 weeks in culture. By that time the leaf tissue had formed irregular masses of callus, less extended than the ones visible in Fig. 7, around the lower edge of the leaf piece. The originally bright green color of the leaf had turned to greenish-yellow, leaving

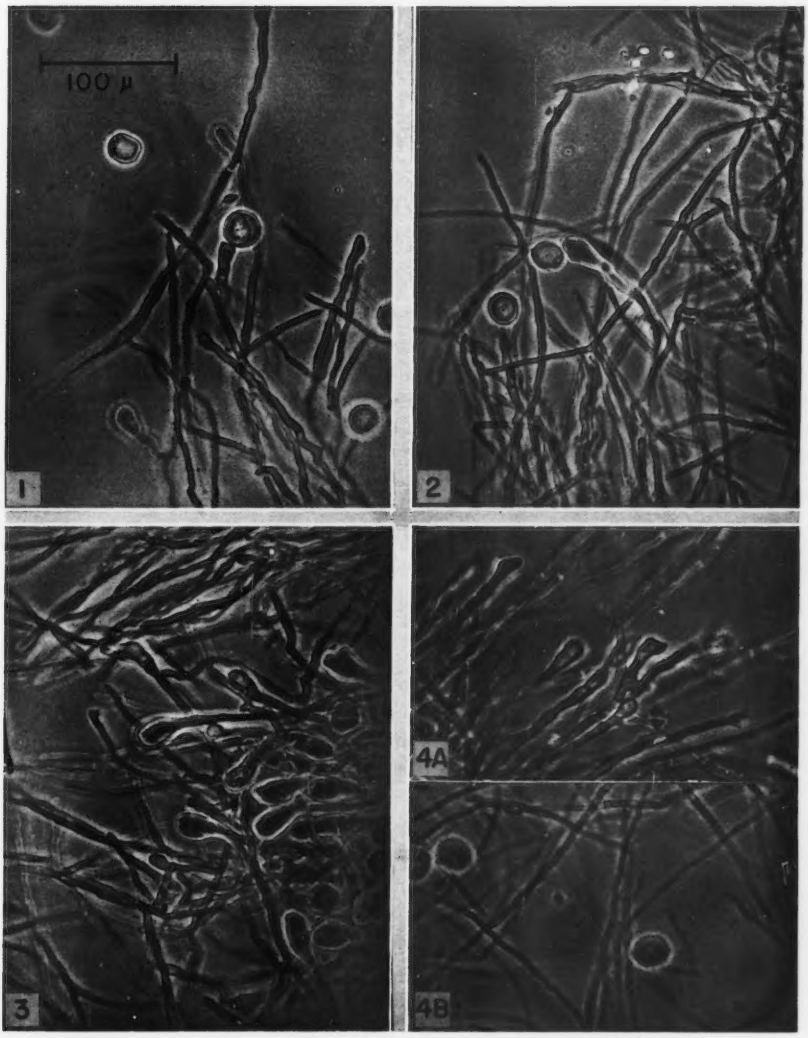

FIG. 1. Aerial mycelium of flax rust from tissue culture of cotyledon showing shedding of a uredospore.
FIG. 2. Aerial flax-rust mycelium showing slightly distorted teliospore.
FIG. 3. Teliospores in different stages of development on aerial flax-rust mycelium.
FIG. 4*a*. Cell fusion at the ends of flax-rust hyphae shortly before spore formation starts.
FIG. 4*b*. Cell fusions between young thin hyphae of flax rust.

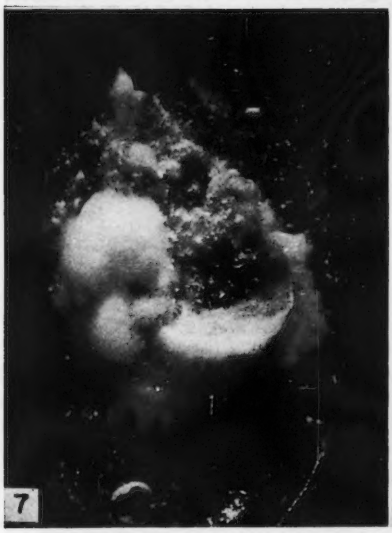

FIG. 5. Section through rust infected flax leaf from tissue culture. Lower surface was in contact with the agar where only uredospore formation took place. Upper surface was opposite the agar where aerial rust mycelium was formed.

FIG. 6. Rust infected flax leaf tissue culture on agar, almost covered by abundant formation of aerial rust mycelium (grown in 16 mm. diameter tubes).

FIG. 7. Formation of aerial rust mycelium and growth of flax leaf callus. Dark spots in the center of the leaf are sub-epidermal teliospores. The glossy bodies at the right and lower edge are leaf callus. The light powdery spots on the upper part of the leaf and on the agar are shed uredospores and the light fluffy mass on the left center part of the leaf is aerial mycelium (grown in 16 mm. diameter tubes).

FIG. 8. Cottony growth of aerial flax rust mycelium on leaf tissue on agar (grown in 16 mm. diameter tubes).

only few spots which contained fair amounts of chlorophyll. The rust formed a dense cover on the exposed adaxial leaf surface, at first white and then turning light orange. The rust hyphae were about 1 to 2 mm. long and 3 to 7 μ in diameter, depending on the age of the mycelium and the sugar content of the medium. The single type of branching predominated (Figs. 1 and 2) but some double branching was observed. Fusions between cells of two neighboring hyphae were quite frequent (Figs. 4*a* and 4*b*). The septa were easily seen even when oil droplets appeared in the cells as the mycelium grew older. The tips of the hyphae were quite characteristically rounded (Figs. 1 and 3) and frequently swollen.

Both uredospores and teliospores were present in all stages of development (Figs. 1, 2, and 3). When spore formation started, the swollen tips were divided from the rest of the hyphae by a septum.

No shedding of teliospores was observed. This is not surprising since, under natural conditions, teliospores develop only subepidermally. (Subepidermal teliospores were frequently formed on leaves in tissue culture (Fig. 7) where they show up as black areas.) The teliospores were often abnormal in shape and have not been observed to germinate.

The uredospores apparently matured normally. In Fig. 1 the shedding of one is nearly completed. These spores germinated when transferred to agar medium not later than 4 weeks after the leaf culture was set up. Percentage germination was, however, lower than with spores grown on host plants under normal conditions.

Leaf sections showed that the intercellular mycelium penetrated the entire leaf and in some places it completely filled intercellular spaces up to 150 μ in diameter. Sometimes characteristic formation of subepidermal teliospores occurred in such densely packed masses of hyphae.

Yield and condition of the mycelium were best when the tissue cultures were about 4 weeks old. After 5 to 6 weeks on agar, the host tissue started to degenerate. Its color turned dull yellowish, the cells began to shrink, and the rust hyphae gradually dried up. Transfer of the cultures to fresh medium did not revive growth.

Mycelial growth occurred on 70 to 100% of the cotyledons (80 to 500, depending on experiment) which were prepared for this purpose but the amount of mycelium produced per leaf varied greatly (Figs. 6 and 7). In several instances, up to 15 mg. of mycelium were harvested from a single culture, but more frequently about 5 mg. were obtained. Where the rust growth was abundant, the hyphae formed thick mats (Fig. 6) which could easily be removed, without contamination by host cells, and could be sliced like pieces of soft mushroom,

The rust growth was the same on secondary leaves attached to short pieces of flax stem in tissue culture. But rusted cotyledons were used in all following experiments because of their larger size.

Rusted flax cotyledons which were not cut, but were placed whole on the medium, rarely developed appreciable aerial rust mycelium, but produced large amounts of mature, powdery uredospores. About 6 to 8 mg. of spores

could be shaken off a whole leaf after 3 to 4 weeks in culture. Physiological studies showed that in spores older than these a marked decrease in respiration had occurred.

Parasite–Host Relationship

When only production of uredospores was the aim, growth of leaf callus was neither necessary nor desirable. The spore yield was optimal on undivided leaves, where there were no cut edges to stimulate callus development. Leaves with well developed pustules about to break open were selected for spore production. Heavily infected leaves were already weakened at this stage and on the tissue culture medium they showed only some general enlargement without cell division, and often curled up to form real "spore pockets".

Mycelial growth, however, depended on the right equilibrium between parasite and host tissue. The latter had to be vigorous enough to produce a fair amount of callus which did not grow too abundantly. The first sign of mycelial growth usually appeared after 1 to 2 weeks, but, in most cases, thick growth started after about 3 weeks. By that time, callus growth was usually slowing down and it seemed that this finally stimulated abundant formation of aerial mycelium.

When callus growth was too vigorous, the aerial mycelium either did not appear at all or remained in its initial short and sparse stage without developing a felt-like abundance. Often it was finally outgrown by the host tissue to such a degree that, after a promising start, it became hardly visible.

Effect of Various Factors on the Development of Callus and Rust Growth Conditions

Liquid media were inferior to solid agar media of the same nutrient content because the leaves tended to sink in the liquid and in the submerged state no mycelium was formed on the leaf surface.

Best results were obtained with Difco Bacto-Agar (No. 0140–01). This agar, washed in running cold water, and Difco Special Agar (Noble), as well as agars less purified than Difco Bacto-Agar, gave poorer results.

The cutting of the leaf edges promoted growth of callus and so did two subsequent treatments with 4% Javex solution (10 and 4 minutes) instead of one. Light was essential for proper development of callus and mycelium (300–600 ft-c.).

Rust growth on flax leaves occurred with a pH range of 3.0–7.5. At pH below 4.0 and above 6.0 only traces of hyphae appeared. At pH levels between 4.5 and 5.6 production of rust mycelium was maximal.

Nitrogen Supply

White's medium was unsuited for growth of the flax tissue, as well as for the development of aerial mycelium, whereas Gautheret's, Knop's, and Heller's media were equally well suited. The only difference between Gautheret's and Knop's media is that the former contains exactly half the amount of all macroelements present in the latter. Since we could not find any difference in response to the two media, we concluded that within certain limits the absolute

ion concentrations were much less important than the relative concentrations. White's medium was the least well balanced of the media used. Most striking is its short supply of nitrate ions. The relation between nitrogen nutrition of the host plants and the rust diseases has interested investigators for a long time (1, 3, 11).

To examine the role of nitrogen in our tissue cultures, a series of experiments was carried out with different concentrations (2.0, 1.0, 0.5, and 0.25 g./liter) of calcium nitrate tetrahydrate in Knop's solution. At the highest concentration of nitrogen, callus growth was more abundant and more regularly distributed over the whole leaf surface and the mycelium was thicker and grew in larger patches than in the controls. However, if the nitrogen content was increased still further by adding 0.8 to 1.6 g./liter of ammonium nitrate to the standard Knop-solution (containing already 1 g./liter of calcium nitrate tetrahydrate), the chlorophyll content in the leaves remained so high and the callus growth was so vigorous that the aerial mycelium soon was outgrown by the host tissue as mentioned above. Sporulation was also drastically reduced.

Heller (5, pp. 136–138) reported that growth of cultures of carrot and other stem and root tissues was impaired on media containing 20 mM. of ammonium ions (0.8 g./liter of ammonium nitrate) or as high concentrations of nitrate ions as were used in our experiment (28.5 mM. of nitrate ions). Our cultures of fresh leaf tissue behaved the opposite way and it is possible that the action of chlorophyll played an important role.

Various organic nitrogen sources were tested. Urea (0.001 *M*) had no effect nor did the following amino acids supplied singly at 0.01 g./liter: glycine ethyl ester hydrochloride, *l*-alanine, *dl*-leucine, *l*-aspartic acid, *dl*-glutamic acid monohydrate, *l*-arginine monohydrochloride, and *dl*-tyrosine. Complex nitrogen sources related mainly to nucleic acids were tested singly at a concentration of 0.001 g./liter of medium without effect: adenosine, adenylic acid, cytidylic acid, desoxyribonucleic acid (sperm), diazouracil, dihydrothymine, glutathione (reduced), guanine (free base), guanosine, guanylic acid, hypoxanthine, isocytosine, orotic acid, protamine nucleinate, protamine sulphate, ribose nucleic acid, thiouracil, thymine (5-methyl uracil), uracil, uric acid, uridine, and xanthine. The addition of 0.01 g./liter of adenine (2) to Knop's medium had no effect on the host tissue but it increased the amount of mycelial growth and the rust hyphae were of a deeper orange color than in the controls.

Carbohydrate Sources

Sucrose or glucose were equally suitable as carbohydrate sources, but the usual concentration of 2%, recommended for tissue cultures, was suboptimal. Callus and mycelial growth increased with increasing amounts of either of the two sugars up to a concentration of 5%. Higher percentages were toxic to the host tissue.

Neither 0.1% pectin nor 1% glycerol had a beneficial effect when added to Knop's medium. Oleic acid (0.5%) in addition to 1% glycerol (10) hindered growth of tissue and mycelium.

The medium of Yarwood and Cohen (14) containing gelatin, lecithin, and fructose caused the leaves to turn pale after a short time in culture. Growth of sparse mycelium started in small areas but stopped soon and the leaf and fungus died. No callus growth occurred. When this medium was added to Knop's solution without agar some callus growth was observed but the rust did not develop.

Plant Hormones and Vitamins

Indole-3-acetic acid (0.1 mg./liter) seemed to stimulate callus and mycelial growth in the presence of the usual concentration (10%) of coconut milk when not more than the standard amount of nitrogen was present in Knop's medium. In the absence of coconut milk, or with ammonium nitrate (20 mg./liter), no such effect could be detected. Maleic hydrazide (1 p.p.m.) (9) did not affect callus and mycelial growth but stimulated formation of black subepidermal teliospores while 5 p.p.m. or more hindered growth of callus and mycelium. The combination of vitamins used for sunflower stem callus tissue cultures by Henderson *et al.* (6) was tried without effect.

Coconut Milk

All observations showed that in the standard Knop-solution, coconut milk was the main factor in promoting a desired amount of callus growth and a satisfactory development of aerial rust mycelium. Therefore differences in quality of the coconut milk were strongly reflected in the behavior of the cultures. Locally purchased ripe nuts usually furnished highly satisfactory milk which was merely passed through a fine cotton cloth to separate it from coarse impurities before use. At first, Seitz filtration was used to sterilize the milk before storing it at −30° C. Passage through a Seitz filter removed most of the turbidity from the fresh coconut milk. This turbidity, caused by small particles, could be separated by ultracentrifugation in the form of a grayish, somewhat slimy sediment. About 100 mg. dry weight of this fraction was obtained from 400 ml. of coconut milk centrifuged at a speed of 25,000 r.p.m. for 20 minutes. Chemical tests showed that the amino acid content of the dried sediment, after hydrolysis, was twice as high as that in the same weight of freeze-dried uncentrifuged coconut milk. The removal of this fraction brought about a reduction in growth activity of the rusted flax leaves. In one experiment with somewhat inferior coconut milk, an addition of 50 mg. of dry sediment to 100 ml. of Knop's medium containing 10% coconut milk resulted in good callus and excellent mycelial growth compared to the fair growth in the controls. Casein hydrolyzate could not be used as a substitute for the sediment.

Optimal Medium

The following additions to Knop's medium have proved beneficial for growth of aerial flax rust mycelium on tissue cultures: Difco Bacto-Agar No. 0140-01 (0.8–1.0%); turbid whole coconut milk from fresh, ripe nuts (10%); calcium nitrate tetrahydrate (500 mg./liter); ammonium nitrate (10 mg./liter); adenine (10 mg./liter); and sucrose (4%). These amounts are not quite optimal

according to the experiments described above, where each compound was tested separately. The effects of the two nitrogen salts were cumulative as were those of optimal sugar content and high grade coconut milk. Consequently, in experiments where every one of these factors was optimal, the balance between host and parasite was again disturbed to the disadvantage of the rust.

Conclusions

The aerial rust mycelium described in this paper was fully dependent upon the living host tissue and the increases and decreases in production of mycelium were accomplished only by affecting the metabolism of the host tissue. The rust hyphae have not shown any tendency to grow saprophytically and they developed only where the leaf was not in contact with the nutrient medium. When the leaf tissue was degenerating, the growth of the mycelium stopped. In spite of these facts, the work reported has useful applications. Rust mycelium and spores can be grown under controlled conditions and collected for respiration and chemical studies free from host material. This makes it possible to determine the effects of many compounds and conditions on the host or the parasite, either separately or combined, and thus to begin a systematic search for the elusive factors which may be involved in obligate parasitism.

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SOME MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF A SOIL BACTERIUM WHICH DECOMPOSES 2,4-DICHLOROPHENOXYACETIC ACID[1]

G. R. BELL[2]

Abstract

A new *Achromobacter* species which decomposed 2,4-dichlorophenoxyacetic acid (2,4-D), apparently to small molecules, was isolated from a soil treated with successive doses of the herbicide. The organism grew poorly or not at all on common laboratory media in the presence or absence of 2,4-D. Investigation of its carbon, nitrogen, mineral, and vitamin requirements in agar containing 2,4-D showed that the best growth stimulants were the dicarboxylic acids of the tricarboxylic acid cycle, bicarbonate, formate, urea, and L-histidine. Calcium or magnesium and probably iron were required for maximum growth. Some aryloxy acids, phenolic compounds, and an ester were tested for their ability to replace 2,4-D as growth substrate or to inhibit growth in the presence of 2, 4-D, and it was found that the ethyl ester of 2,4-D and chlorophenolic substances were most toxic. Only 2-methyl-4-chlorophenoxyacetic acid (MCPA) and less readily, 4-chlorophenoxyacetic acid, phenoxyacetic acid, and resorcinol could substitute for 2,4-D. Good cell multiplication and herbicide decomposition were obtained in an aerated mineral salts medium containing 2,4-D, yeast extract, and 0.005 *M* L-malic acid. Maximum growth (*ca.* 10^9 cells/ml.) occurred in 4 to 5 days and 2,4-D decomposition was essentially complete in 6 to 7 days. Resting cells were able to oxidize 2,4-D, MCPA, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2,4-dichlorophenol (2,4-DCP) and to release 94% of the 2,4-D chlorine as chloride. High concentrations of yeast extract caused growing cells to accumulate 2,4-DCP.

Introduction

The toxicity of the selective herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is not cumulative because the herbicide undergoes a slow inactivation in soil and this inactivation has been shown by numerous workers to be due principally to biological decomposition (39, 30, 20, 16, 2). Although many workers have isolated different species of bacteria capable of decomposing 2,4-D (3, 4, 5, 6, 40, 29, 55, 22, 51, 44, 45, 53, 52), biological and biochemical information concerning these organisms is still meager because of inadequate investigation of the nutritional requirements of the isolates and a consequent failure to obtain sufficient yields of consistently active cells for physiological studies. In the present study a bacterium which decomposes 2,4-D has been isolated and described in some detail, and media in which it grows well and maintains its ability to decompose 2,4-D have been developed.

Methods

Quantitative Estimation of 2,4-D

(*a*) *Chromotropic Acid*

Since it is proposed to publish details of the method elsewhere, only an outline will be presented here. The method involved a special type of extraction of 2,4-D from soil extracts or from other fluids using ethyl ether, following

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Contribution No. 101 from the Science Service Laboratory, London, Ontario. Taken, in part, from a thesis submitted to the Faculty of Graduate Studies of the University of Western Ontario, London, Ontario, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Some of this work was presented at the Sixth Annual Meeting of the Canadian Society of Microbiologists, Macdonald College, Ste. Anne de Bellevue, Quebec.
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which the ether was evaporated and the residue heated with 1,8-dihydroxy-naphthalene-3,6-disulphonic acid (chromotropic acid) in sulphuric acid according to the method of Le Tourneau and Krog (33). In the presence of 2,4-D a mauve color (24) was produced which obeyed Beer's Law over the concentration range used. Calibration curves prepared simultaneously with the samples were used as a basis for quantitative estimation of 2,4-D. In spite of the low specificity of the reaction (24) there were remarkably few interferences even from high organic matter soils.

(*b*) *Ultraviolet Light*

Where possible, 2,4-D was also determined by the ultraviolet light method of Bandurski (9). The 283 mμ absorption maximum was used to establish daily calibration curves on which estimates of 2,4-D concentration were based. Bandurski found the k value of 2,4-D (25μg./ml., 283.5 mμ) in water to be independent of reaction over the range pH 2.2 to 8.0.

The terms 'decomposition' and 'degradation' rather than 'detoxication' will be used throughout this work because chemical instead of biological methods have been used to estimate 2,4-D.

Selective Enrichment of Soil Populations

It was necessary to increase the numbers of organisms in soil capable of decomposing 2,4-D before attempting any isolations. For this purpose it was found most convenient to add 10 mg. of 2,4-D as an aqueous, neutralized solution to 50–75 g. of air-dried soil in a 500 ml. Erlenmeyer flask. Successive doses were added whenever the 2,4-D disappeared. A soil high in organic matter was used throughout this work because preliminary data showed it to be more active than soil low in organic matter in decomposing 2,4-D. The soil was collected from a grassy slope on which young spruce trees were growing. There was no history of herbicide treatment. Soil was also selectively enriched using a modified Audus (1) perfusion apparatus. It is of interest that the kinetics of decomposition of 2,4-D in soil shown by Audus (2, 4) has been substantiated using a chemical rather than a biological assay.

Media and Isolation

The initial isolation of bacteria from the selectively enriched soil population was done by streaking on a variety of mineral media containing 2,4-D as the principal carbon source. A predominant colonial type, designated Bacterium R, was selected for study after it was established that the bacterium could decompose 2,4-D in sterile soil, grew much better in the presence than in the absence of 2,4-D (Fig. 1*A* and *B*), and increased its oxygen uptake in the presence of 2,4-D. The addition of 0.01% (w/v) L-glutamic acid (43) to various mineral media containing 2,4-D increased the growth but even the best growth was only a thin film. The best basal medium, designated Medium 23, was used extensively throughout this work. It had the following composition in g./liter: 2,4-D (Eastman Kodak, White Label, m.p. 139–140°C., uncorrected), 1.0; NH_4Cl, 0.3; K_2HPO_4, 0.1; $CaCl_2$, 0.2; $MgSO_4.7H_20$, 0.2;

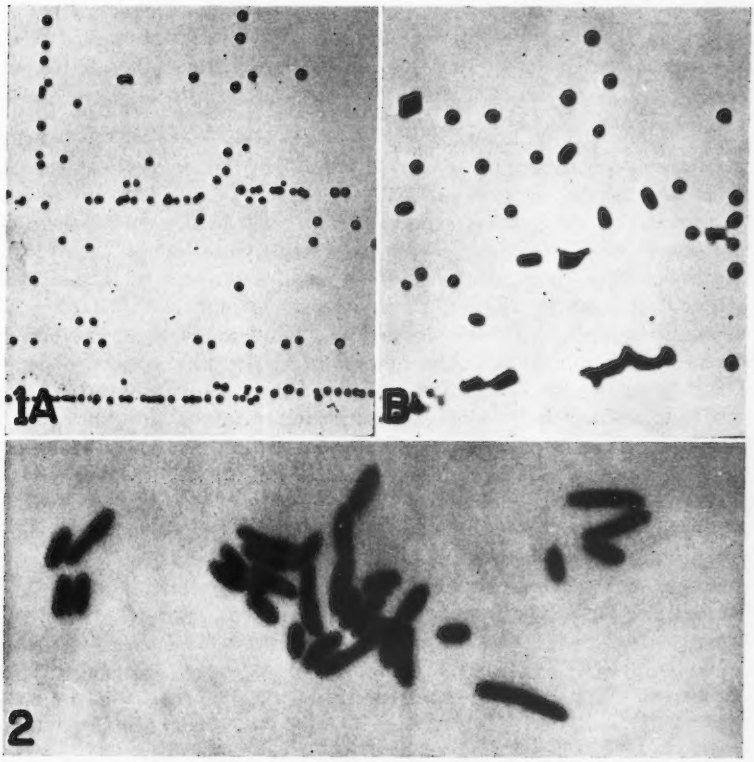

FIG. 1*A*. Bacterium R grown 6 days on soil extract medium (35) with 0.01% L-malic acid, pH 7.0. Photographed using transmitted light. ×3.5

FIG. 1*B*. Same as Fig. 1*A* but with 0.1% 2,4-D. ×3.5

FIG. 2. Bacterium R grown 5 days on Medium 23 containing 0.01% L-malic acid, pH 7.0. Stained 10 minutes with ammonium oxalate–crystal violet of Gram's stain. ×3600

$FeSO_4.7H_20$, 0.005; agar, 15. The reaction was adjusted to pH 6.8–7.0 and the medium was autoclaved at 10 lb. for 15 minutes. Medium X23 was similar to Medium 23 except that 2,4-D was omitted.

The nutritional study reported below had two main purposes: first, to find more effective growth stimulators than glutamate, and second, to demonstrate in a broad way the nutritional pattern and physiological behavior of this organism.

Testing and Evaluation of Solid Media

Aqueous stock solutions, pH 7, of the test substances were prepared at concentrations of 1% (w/v) for solids and 1% (v/v) for liquids: all percentage concentrations were w/v or v/v. The solutions were sterilized by autoclaving at 10 lb. for 15 minutes or if there was even slight danger of decomposition, by filtration through sintered glass. The solutions were added aseptically to the melted, cooled mineral base to give a final concentration of 0.01% (w/v or v/v) of the free compound, unless otherwise stated. When solubilities were limiting, saturated stock solutions were added at 1% (v/v) to the basal medium. Most substances were tested at a final concentration of 0.01% mainly because this concentration was commonly employed by Lochhead and co-workers in their nutritional studies of soil bacteria (37, 56). The inoculum consisted of one drop of an aqueous suspension of 5-to 7-day-old cells grown on Medium 23 plus glutamate. Medium 23 containing 0.01% glutamic acid was used as a standard and the amount of growth on duplicate plates of other media related to this. The optimum range for growth was 28–30° C.: incubation was at 28° C. Smears of the growth were examined principally to make sure that gum production was not mistaken for cell multiplication. Since the results were semiquantitative, no attempt has been made to assign numerical values to the amount of growth. Substances which appeared to be inhibitory were evaluated by their effect upon growth on otherwise nutritious media.

Testing and Evaluation of Liquid Media

The nutritional properties of liquid media were usually tested by dispensing 10 ml. of medium into 50 ml. Erlenmeyer flasks. After inoculation and incubation, the growth was measured by determining the turbidity at 420 mμ in the Unicam spectrophotometer (Unicam Instruments, Cambridge, England).

Results

Decomposition of 2,4-D in Soil

The main intention of this work was to isolate bacteria capable of decomposing 2,4-D but, incidentally, some observations of other workers were confirmed using a newly developed chemical method for the estimation of 2,4-D. It was first of all confirmed that microorganisms are responsible for the degradation of 2,4-D in the soil. Effective populations were developed by using percolators, trickling filters, and soil in Erlenmeyer flasks and it is important to recognize that the nature of both the physical and chemical

environment might influence the type of organism developed. It is probable that microorganisms other than bacteria can decompose 2,4-D but most workers have only been interested in isolating bacteria. The results (10) of incubating soil under anaerobic conditions indicate, in agreement with those of Steenson and Walker (52), that only aerobic microorganisms are capable of proliferating and decomposing 2,4-D but is it possible that the products of an anaerobic degradation of 2,4-D may have interfered with the estimation of the herbicide.

Characteristics of a 2,4-D Decomposing Isolate—Bacterium R

Colonies

Bacterium R was isolated on June 26, 1954, from an Erlenmeyer flask of soil which had decomposed 2,4-D. Its colonial characteristics are illustrated in Fig. 1*A* and *B*. The results are typical of other media but a soil extract medium was chosen as more 'natural'. The colonies were translucent-white, round, entire, and flat. Subsurface colonies were pinhead size, cream colored, lens- and oval-shaped. Both surface and subsurface colonies developed dark centers after *ca.* 2 weeks' incubation.

Cells

The description of the bacterium refers to cells grown for 5–7 days on Medium 23 containing 0.01% L-malic acid but its morphology was remarkably constant. One- to two-month-old cultures contained a small proportion of swollen cells but no pleomorphic forms were seen except on smears from amino acid media. Unless otherwise indicated, the staining methods were those recommended by Conn, Bartholomew, and Jennison (18).

The organism is a slightly irregular rod 1–2 μ × 0.3–0.5 μ (Fig. 2), motile, Gram-negative, asporogenous, and non-acid fast. Occasional very short rods have been observed. Cells appeared to be non-capsulated (Anthony's Method, Tyler's modification) but capsules showed on the flagella stain of Leifson (32) and occasionally on nigrosin smears. The organism had monopolar and dipolar granules when stained with "methylene blue in dilute alcohol" but none according to Albert's diphtheria stain. One of the most interesting features of the cell was a polar fat body which stained with Sudan black. Eleven-day-old cells were similar but showed traces of fat throughout. The various flagellar types are shown in Fig. 3; the most common appeared to have two laterally placed flagella but electron microscopy (Fig. 4) revealed a further common type having lophotrichous flagellation. The types resemble those of *Mycoplana bullata*, illustrated by Gray and Thornton (26).

Biochemical Characteristics

Carbohydrate fermentation tests were performed using the technique of Smith, Gordon, and Clark (48) but the basal medium consisted of Medium X23 (i.e., no 2,4-D) with 0.01% Difco yeast extract. The natural isomers of the carbohydrates were used. Bacterium R grew on all media but especially well on the pentoses. Acid was formed in 1–2 weeks from lyxose, arabinose, ribose, and xylose. Galactose and mannose were not fermented until 2–3

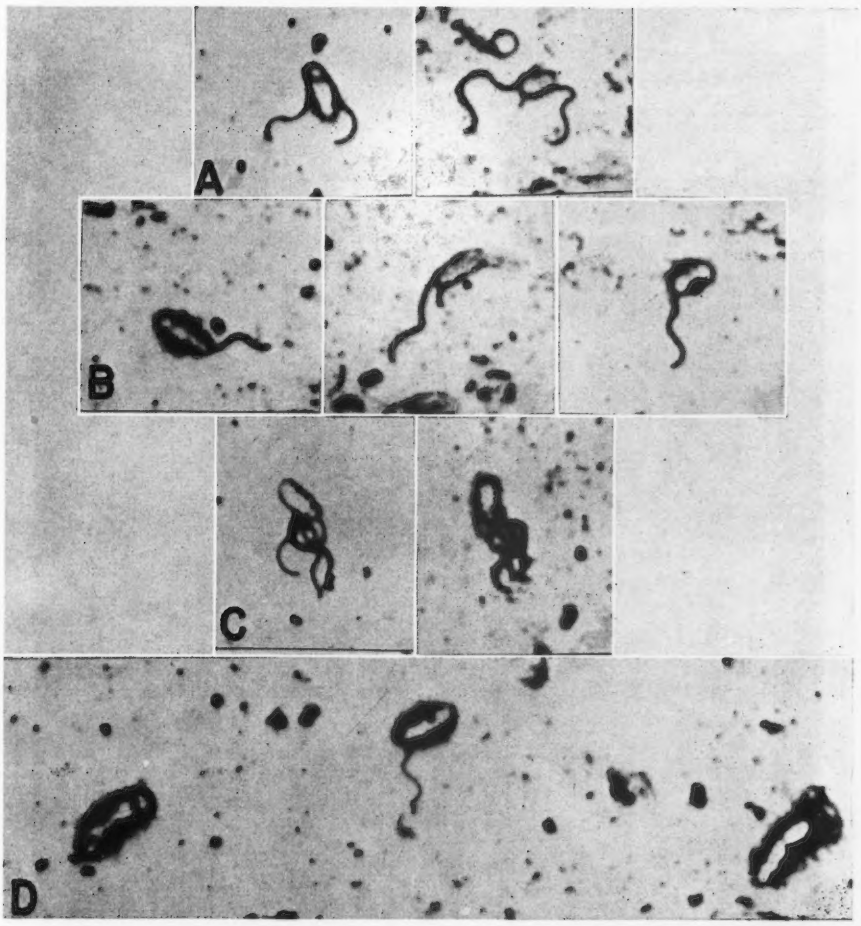

FIG. 3. Leifson (32) flagella stain of a 5-day-old culture of Bacterium R grown on Medium 23 with 0.01% formic acid, pH 7.0. ×3500 (*A*) Most common types. (*B*) Next most common. (*C*) Rare; some flagella may only have adhered to the capsular material. (*D*) A monotrichous type (center) and two cells showing strands of gum.

FIG. 4. Electron micrograph of a shadowed cell from a 3-day-old culture of Bacterium R grown on Medium 23 with 0.005 *M* L-malate. The round bodies along one side of the cell might be artifacts or aggregations of capsular material. ×50,000

weeks. There was no acid produced in 3 weeks from the following: cellobiose, dextrose, dulcitol, lactose, laevulose, maltose, mannitol, melibiose, raffinose, sorbitol, sorbose, starch, sucrose, and trehalose. The fermentation of pentoses, galactose, and mannose, most of which have been found in hemicelluloses, suggested that the organism might be active in decomposing hemicelluloses in soil but it apparently did not decompose a xylan or any of several hemicelluloses in laboratory media. This failure may have been due to an inadequacy on the part of the basal medium rather than the organism, as can be said of many nutritional data.

Bacterium R did not hydrolyze starch, dextrin, or cellulose nor did it decompose gelatin or casein. Indole was not produced from either indoleacetic acid or tryptophan. Evidence that the organism can denitrify will be presented in the section concerning liquid media. The organism did contain a urease, according to the method of Smith *et al.* (48). In litmus milk there was reduction of the litmus in 10–12 days; the oxidized litmus was slightly alkaline but the milk was otherwise normal. There was a moderate amount of growth in the milk and the cells appeared typical.

Classification of the organism according to the nutritional grouping system of Lochhead and Chase (35) was also attempted. Only media B, A, AG, Y, and YS were used: the amino acid mixture was substituted with 0.4% Bacto vitamin-free casamino acids (Difco) and the growth factor mixture included vitamin B_{12} at 2 μg./liter (34, 36). The inoculum was cultivated on a mineral salts, 2,4-D medium. Bacterium R gave 4+ growth in YS and no growth in B and Y. Inocula which had been cultivated on nutrient agar responded to the yeast extract (2+ growth) as well as to the amino acids. The results indicated that the organism belonged to the amino acid requiring Group II of Lochhead and Chase (35), an obviously untrue conclusion. This organism was not amenable to classification by this nutritional system because it apparently does not utilize dextrose and it is inhibited by 0.1% yeast extract but the results are important in that they further reveal the nutritional pattern of the cells and point out some possible limitations of the grouping system.

Nutritional Requirements of Bacterium R on Solid Media Containing 2,4-D

The organism grew poorly on nutrient agar (Difco) and potato dextrose agar (Difco) but not at all on heart infusion agar (Difco) or on an agar made from nutrient broth (Oxoid), each with and without added 2,4-D. Growth on nutrient agar was granular; young cells were typical but older cells were often shrunken at one end. The growth was considerably improved by continual subculture. Heaviest growth occurred when special agar, Noble (Difco) was used as a solidifying agent, but ordinary Difco agar was used, unless indicated. Difco agar which had been extracted with methanol–ether to remove fatty acids was no more effective in allowing growth than was the unextracted agar.

Growth was almost unaffected by reaction between pH 5.5–8 but since the growing organism lowered the pH, most media were adjusted to pH 6.8–7.0.

The optimum 2,4-D concentration was 0.1–0.2%; inhibition began at 0.4% and growth was stopped at 0.6% 2,4-D, in agreement with the work of Jensen and Petersen (29) and Spicher (49).

Effect of Carboxylic Acids on Growth

The growth promoting activity of various carboxylic acids can be assigned conveniently to three groups: inferior to glutamate, citric acid, and fumaric acid (0.1%); equal to glutamate, adipic acid, fumaric acid (0.001%), DL-gluconic acid, DL-lactic acid, DL-muconic acid, oxalic acid, and succinic acid; superior to glutamate, *cis*-aconitic acid, formic acid, fumaric acid, D-*iso*citric acid, α-ketoglutaric acid, oxaloacetic acid, oxalosuccinic acid, L-malic acid, pyruvic acid, succinic acid, and tartaric acid (British Drug Houses). Because of the instability of oxalosuccinate, *cis*-aconitate, and especially oxaloacetate, any interpretation of their function is uncertain. The most stimulating compounds were the dicarboxylic acids of the tricarboxylic acid cycle. Citric acid was a very poor carbon source and the organism would not grow on the citric acid medium of Smith *et al.* (48). Mixtures of dicarboxylic acids of the tricarboxylic acid cycle were only slightly more effective than the acids alone. The relative growth promoting activity of equimolar concentrations of some carboxylic acids is shown in Table I. The members of the tricarboxylic acid cycle, exclusive of citrate, still appear to be the best growth promoting compounds. Glycollic and glyoxylic acids were tested to see if these possible products from the cleavage of the 2,4-D side chain acted as inhibitors. The utilization of these acids, as indicated by increased alkalinity, was masked by acid produced apparently from 2,4-D. However, the pH value increased when cells were grown on Medium X23 containing either fumaric, glycollic, or glyoxylic acids whereas a control of X23 remained at *ca.* pH 7. Although the growth was poor, the results indicated that glycollic and glyoxylic acids were utilized and should not accumulate from the breakdown of 2,4-D by this organism.

TABLE I

RELATIVE GROWTH PROMOTING PROPERTIES OF EQUIMOLAR QUANTITIES OF CARBOXYLIC ACIDS

Compound	Ranking	
	4 days	8 days
Acetate	5	4
Citrate	4	4
Formate	3	4
Fumarate	2	2
Glycollate	4	4
Glyoxylate	4	4
α-Ketoglutarate	1	2
L-Malate	1	1
Pyruvate	3	3
Succinate	2	3
Tartrate	4	4
None	5	5

All concentrations $10^{-3}M$. Ranked 1 to 5, heaviest to lightest growth. Average of duplicates.

As a result of these and other investigations reported below, Medium 23 containing 0.01% (acid equivalent) fumarate, succinate, L-malate, or formate has been adopted for the maintenance of the organism. Formate was often used because it could be autoclaved conveniently in the medium. These media realized an objective of the nutritional study in that they gave good growth of the organism during *ca.* 3 years without causing loss of vigor of either growth or metabolism of 2,4-D.

Effect of Growth Factors on Growth

The following vitamins were added as a mixture to Medium 23 and to 23 plus formate to give 1 and 10 times the concentrations indicated in μg./liter of medium: biotin, 2; folic acid, 10; nicotinic acid, 100; pantothenic acid, 50; *p*-aminobenzoic acid, 100; pyridoxal, 50; riboflavin, 100; and thiamine, 50. The lower concentration of vitamin mixture was without effect but the higher concentration was inhibitory. Of the individual vitamins, only nicotinic acid, pyridoxal, and riboflavin caused any increase in growth and this increase was modest. The following growth factors were also found to be without effect: *i*-inositol, (0.01%); glutathione, 0.5 μg./ml.; pimelic acid, 0.1 and 0.01 μg./ml.; and calcium phytate, 0.01%. Vitamin B_{12} (Nutritional Biochemicals), 0.01 μg./ml., was stimulatory only when formate was also added. In general, the organism was not responsive to the addition of growth factors.

Effect of Simple Nitrogen Sources on Growth

Growth was unaffected by guanidine and KNO_3 (0.02%), inhibited by $NaNO_2$ (0.02%), and stimulated by urea. The efficacy of some nitrogenous compounds was tested by adding them aseptically to Medium 23 plus fumarate, minus NH_4Cl, to give a nitrogen concentration equivalent to that in 0.01% urea. Interpretation of the results shown in Table II is complicated by the presence of various anions but it is clear that ammonium ion is the best source of nitrogen for growth. When the organism was serially transferred

TABLE II

RELATIVE GROWTH PROMOTING PROPERTIES OF SIMPLE NITROGEN SOURCES OF EQUAL NITROGEN CONCENTRATION TO 0.01% (W/V) UREA

Compound	Ranking*	
	4 days	8 days
Urea	2	3
KNO_3	5	5
NH_4Cl	1	1
$(NH_4)_2HPO_4$	3	2
$(NH_4)_2CO_3$	4	4
NH_4OH	7	Sterile 7
None	6	6

*Ranked 1 to 6, heaviest to lightest growth. Average of duplicates.

10 times during 3 months on Medium 23 plus fumarate minus NH_4Cl, the growth was greatly reduced and the medium turned alkaline. In later transfers the growth increased, indicating that the organism could get its nitrogen, probably as NH_3, from the agar or the atmosphere.

Effect of Amino Acids on Growth

The growth promoting properties of various amino acids compared to those of glutamate were as follows: inferior, L-cysteine, glycine, DL-norvaline, and L-threonine; equal, β-alanine, DL-α-aminobutyric acid, L-arginine, L-aspartic acid, L-hydroxyproline, DL-isoleucine, DL-lysine, L-methionine, DL-ornithine, L-proline, DL-serine, and L-tyrosine; superior, L-α-alanine, DL-α-aminoadipic acid, L-asparagine, DL-citrulline, L-glutamine, L-histidine, L-leucine, L-phenylalanine, L-tryptophan, and DL-valine. Growth was inhibited by L-cysteine and glycine and markedly stimulated by L-histidine. Glycine caused the appearance of granular rather than the normal mucoid growth but it was not so inhibitory in the presence of other amino acids.

Bacterium R grew poorly on a mineral medium containing ammonium ion, fumarate, and mixtures of amino acids, exclusive of cysteine, with and without added 2,4-D and glycine. Growth was more sparse when 2,4-D was omitted from the media. On the amino acid media the organism showed a requirement, not demonstrated on other media, for the metal complex of Hutner, Provasoli, Schatz, and Haskins (28). It is unlikely that the ethylenediaminetetraacetic acid (EDTA) of the metal complex was required to remove toxic metal contaminants from the amino acids because the EDTA was inhibitory at concentrations over 0.0001% when substituted for the metal complex. The inhibitory effect of the amino acids, which is possibly due to chelation of metals, could not be reversed by the addition of calcium, iron, or magnesium. Cells from the amino acid media were pleomorphic, consisting of long and short rods and gourd and marrow-shaped types.

Effect of Purines and Pyrimidines on Growth

The organism grew as well with the addition of thymine, uracil, cytosine, adenine, and guanine, each at 0.001%, as with glutamate. A mixture of the purines and pyrimidines gave similar results.

Effect of Carbohydrates on Growth

The following natural isomers of carbohydrates added aseptically to the basal medium caused no more growth than did glutamate: arabinose, cellulose, bacterial dextrin, dextrose, glucuronolactone, lactose, laevulose, mannitol, ribose, sucrose, and xylose. The following stimulated growth more than did glutamate: cellobiose, galactose, dextrose (autoclaved in the medium), glycerol, pectin, and soluble starch. Cells grown on cellobiose presented a strikingly uniform morphology: Gram-negative, evenly stained, short rods with one end diffuse appearing. Their morphology was similar to that of *Cellfalcicula fusca* shown on Plate XXIV, Fig. 1, No. 6 by Winogradsky (57).

Effect of Fatty Acids and Esters on Growth

Growth on Medium 23 plus formate was inhibited by acetic, propionic, *n*-butyric, and *n*-valeric acids. "Tween 81" (0.001%), ethyl malonate (0.001%), and oleic acid at 2–20 μg./ml. had no effect but ethylacetoacetate at 0.01% was stimulatory.

Effect of Minerals on Growth

Despite the fact that agar contains appreciable amounts of free and combined metals (23, 7, 41), some attempts were made to demonstrate the metal requirements of the organism or at least the effect of various metal ions on growth. Bacterium R grew as well, following 10 serial transfers during 3 months, on Medium 23 plus fumarate (solidified with Noble agar) as on similar media which had in turn each mineral constituent missing. Nevertheless, the bacterium has been maintained on Medium 23 because it grew well on the complete medium with a minimum production of gum. The effect of nutrients upon gum formation was shown by Duguid and Wilkinson (21), who found that a deficiency of nitrogen, phosphate, and sulphate in media caused a marked increase in polysaccharide production by *A. aerogenes*. We have not subjected Noble agar to an elemental analysis but it did not contain any readily water soluble phosphate. Either the phosphate requirement was extremely low or bound phosphate was released chemically or enzymically.

The minerals of the "A-Z" trace element mixture of Hoagland and Snyder (27) were added aseptically as individuals and as the complete mixture to Medium 23 plus formate to give 0.1, 1, and 10 times the final concentration used by Meiklejohn (38). None of the additions had any effect. A wide variety of minerals was also provided in the form of pure, unsoftened tap water used as a diluent in Medium 23 but the growth was not improved.

Growth on Medium 23 plus formate was strongly inhibited at a minimum concentration of 0.05 m*M* α, α′-dipyridyl, 8-hydroxyquinoline, phenylthiourea, and EDTA, indicating that the cells required for growth, iron, and some or all of the following: Mn^{++}, Zn^{++}, Co^{++}, and Cu^{++}. As will be mentioned later, the organism was shown to require added calcium or magnesium in liquid media. The complexing agents sodium metaphosphate and sodium pyrophosphate had no effect on growth.

Many bacteria, including Bacterium R, grow better on water washed agars such as Noble's than on the usual commercial agars but the reasons for this are obscure. Ayres, Mudge, and Rupp (7) and Noble (41) attributed the beneficial effect of washing agar with water to the removal of calcium and magnesium salts but this explanation does not apply to the present work because both calcium and magnesium were added to the Noble agar. It is possible that the inhibitory agents in commercial agar may actually be in the 1.5% "protein" fraction but unfortunately Ayres *et al.* (7) did not investigate the effect of washing upon this fraction.

Effect of Complex Additions on Growth

Peptones were added to Medium 23 plus formate and the growth was stimulated by proteose peptone (Difco) but unaffected by peptone (BDH),

tryptose (Difco), Neopeptone (Oxoid), and Lablemco (Oxoid). Skim milk was a stronger growth stimulant than glutamate but vitamin-free casamino acids (Nutritional Biochemicals Co.) at 0.05% were equal in growth activity to glutamate, as were the amino acids plus 0.01% yeast extract. The casamino acids caused the granular growth also observed in the presence of glycine. Yeast extract (Difco) was inhibitory in Medium 23 plus formate but its effects varied from slight inhibition to stimulation on Medium 23 plus glutamate; 0.10% yeast extract was strongly inhibitory in both media. Growth was restricted to a thin film but in 2–3 weeks, large (2–5 mm.), white, round, flat, mucoid colonies appeared scattered over the film. The cells from both types of growth looked identical but the possibility of contamination has not been eliminated. The large colonies may have arisen by dissociation.

Effect of Gaseous Environment on Growth

Growth was inhibited in the presence of either 10% (v/v) carbon dioxide or 95% (v/v) oxygen with 5% (v/v) carbon dioxide. Re-incubation of the plates in the air caused normal growth. (Sodium bicarbonate stimulated growth in air.)

Nutritional Requirements of Bacterium R in Liquid Media Containing 2,4-D

Most of the preceding nutritional study was repeated using liquid media and the results were generally similar. In contrast to the *B. globiforme* of Audus (5), Bacterium R did not respond to the addition of traces of agar to the medium. Usually, growth of Bacterium R caused a drop in the pH to 5–5.5: the addition of calcium carbonate to the medium inhibited the growth.

Although the organism is highly aerobic, aeration of media with normal and carbon dioxide enriched air failed to increase the growth. However, it has recently been found that aeration greatly increased the growth when 0.01% yeast extract was added to the basal medium and the concentration of any one of the stimulatory dicarboxylic acids was raised to 0.005 *M* or *ca.* 0.05% (0.1% was inhibitory). Such a medium will be referred to as Medium 23Y followed by the first letter of the added acid; M(malic), S(succinic), etc.

The results of experiments in which yeast extract was replaced by vitamins or vitamin mixtures indicated that the organism required cofactors in yeast which were qualitatively or quantitatively different from those vitamins tested. Growth but not the extent of 2,4-D decomposition was further increased by the addition of 0.01–0.05% xylose, lyxose, arabinose, or ribose but the pentoses have not been added to routine cultural media because of the possibility that the organism might become 'de-adapted' to 2,4-D.

The specificity of the enzyme system responsible for 2,4-D utilization was investigated by growing cells in 2,4-D-free media containing structural analogues of the herbicide and in media containing both analogues and herbicide. Various other aromatic compounds were tested similarly to find their toxicity or availability to the cells. The growth was measured turbidimetrically at the end of 6 days and after correction for color interference,

the results shown in Table III were determined by relating the growth to that in appropriate control media. MCPA was the only compound which could replace 2,4-D at normal substrate levels but several compounds appeared able to replace 2,4-D at lower levels. It is possible that these compounds only increased succinate utilization. Many of the compounds showing a weak effect might be able to replace 2,4-D if they were fed at non-toxic levels or if they could be prevented from undergoing non-biological degradation. 2,4-DCP is one of the most toxic phenols, having a phenol coefficient of 13. The toxicity of the chloro- and nitro-phenols or their oxidation products is apparent. Still more effective were resorcinol and 4-chlororesorcinol (or their oxidation products), the latter found by Kull, Grimm, and Mayer (31) to be a powerful tyrosinase inhibitor. The high toxicity of the ethyl ester of 2,4-D might be attributed to physical phenomena related to its oily nature rather than to action at a specific site. Compounds which appeared to act in the dual role of substituting for 2,4-D and inhibiting growth in the presence of 2,4-D might have affected specifically the 2,4-D utilizing system.

TABLE III

REPLACEMENT OF 2,4-D AS GROWTH SUBSTRATE AND INHIBITION OF GROWTH IN 2,4-D MEDIA BY CERTAIN AROMATIC COMPOUNDS

Compound	Replacement* at:			Inhibition† at:			
	0.1%	0.01%	0.001%	3 m*M*	1 m*M*	0.1m*M*	0.01 m*M*
2,4-D	+	+	0	0	0	0	0
MCPA	+	+		0		0	
2,4,5-T	−	±		+		0	
2,6-D	0	0		0		0	
Na 2-(2,4-dichlorophenoxy) ethyl sulphate	0	0		±		0	
β-(2,4-Dichlorophenoxy) propionic acid	−	±		+		0	
2,4-Dichloro-6-hydroxyphenoxyacetic acid (17, 22)		0	0		0		0
2-Chlorophenoxyacetic acid (14)	−	±		±		0	
4-Chlorophenoxyacetic acid (14)	−	+		+		0	
Phenoxyacetic acid (14)	−	+		+		0	
Phenylacetic acid	−	0		±		0	
Catechol		−	0		±		0
Resorcinol		+	±		+		+
4-Chlororesorcinol (31)		−	+		+		+
2,4-DCP (5)		−	±		+		0
2-Chlorophenol		−	±		+		0
4-Chlorophenol		−	+		+		0
Phenol		−	+		0		0
2,4-Dinitrophenol		−	±		+		0
Ethyl ester of 2,4-D		−	−		+		+

*Basal medium, X23YS.

†Basal medium, X23YS with 3 millimolar (m*M*) 2,4-D.

KEY: + Effective, > 10%. ± Weak effect, 5–10%. 0 Ineffective. − Growth less than in Medium X23YS.

INOCULUM: washed young cells grown in Medium 23YM. All media in duplicate. Cultures agitated on reciprocal shaker.

The efficacy of some carboxylic acids in promoting growth is shown in Table IV. The degradation of 2,4-D in the cultures probably was masked to a certain extent by the loss or gain of ultraviolet light absorbing substances such as fumarate and the growth was masked to some degree by capsular material. However, α-ketoglutarate appeared to be the most effective growth stimulant but there was little difference among the other dicarboxylic acids.

Although citrate was less effective than the dicarboxylic acids in stimulating growth, it was more effective in stimulating the 2,4-D decomposing activity of the cells. Apparently the organism produced acids from citrate whereas there was a loss of acidity owing to the metabolism of the other acids. Acetate, which at *ca.* 10^{-3} *M* inhibited growth on otherwise nutritious media, and formate had little effect on growth under these conditions but formate may have lost its effect because the concentration was too high (0.005 *M* = 0.023%). The effective concentration of the various acids obviously is critical.

TABLE IV

STIMULATION OF GROWTH AND 2,4-D DECOMPOSITION BY EQUIMOLAR CONCENTRATIONS OF CARBOXYLIC ACIDS IN LIQUID MEDIUM 23Y

Compound	Turbidity at 420 mμ, %T	O.D. at 283 mμ		Final pH
		Sterile controls	Test	
Citrate	62	0.330	0.00	5.2
L-Malate	59	0.335	0.032	7.3
Acetate	98	0.340	0.252	6.3
Fumarate	56	0.383	0.041	7.4
Succinate	57	0.350	0.027	7.5
Formate	78	0.338	0.260	8.4
α-Ketoglutarate	51	0.338	0.022	6.8
Inoculated basal medium (23Y)	81		0.296	4.7
Basal medium		0.351		6.4

50 ml. medium in 200 ml. Erlenmeyer flasks; acids added aseptically from autoclaved, neutral solutions to give 0.005 *M* final concentration. Flasks shaken on reciprocal shaker for 10 days. All readings average of triplicates. Turbidity read vs. inoculated basal medium: inoculated basal medium read vs. water blank. Cultures centrifuged and diluted 1/25 for ultraviolet light examination.

Of the acids, malic was used most frequently in culture media because it gave less gum and more uniform and undistorted cells. Some of the other acids may be equally effective. A growth curve of Bacterium R in Medium 23YM and the kinetics of 2,4-D decomposition are presented in Fig. 5. It can be seen from both curves (ultraviolet and chromotropic) that maximum decomposition of 2,4-D began near the end of the log phase of growth and the reaction was nearly complete in 7–8 days.

The amount of growth in Medium 23YM shake cultures in which ammonium ion was replaced by nitrate ion indicated clearly that nitrate enhanced the growth of the organism, but attempts to demonstrate nitrate reduction failed. However, when heavy suspensions of cells were incubated aerobically or anaerobically with nitrate solutions, nitrite rapidly accumulated. Greater amounts of nitrite accumulated anaerobically than aerobically either because of a more rapid reduction or because of a blockage of further reduction. Nitrite at 0.002 *M* completely inhibited growth.

Bacterium R required added calcium or magnesium but not iron for maximum growth in Medium 23YM. Inhibition of growth on agar by α,α′-dipyridyl indicated that the organism required iron for growth but there was probably enough contaminating iron to allow maximum growth in Medium 23YM.

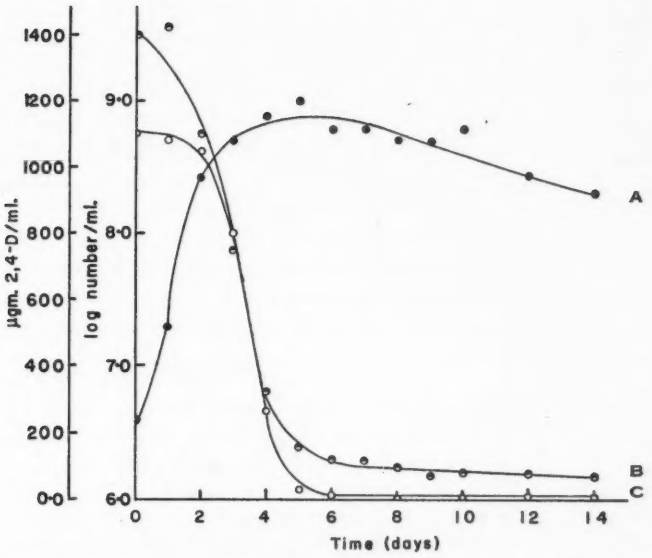

FIG. 5. Growth curve of and 2,4-D decomposition by Bacterium R in Medium 23YM. Viable count (A), average of duplicate plates from duplicate, aerated flasks at 28° C. Concentration of 2,4-D in centrifuged, cell-free medium according to ultraviolet light method (B) and chromotropic acid method (C). Both, average of duplicate samples from duplicate flasks.

If large numbers of cells were needed, the organism was grown for 5 days in Roux bottles of Medium 23YM or S, inoculated into Florence flasks of similar medium, and aerated through a sintered glass sparger. The culture foamed heavily during 3–5 days and ceased thereafter. Yields were *ca.* 1 g. wet weight of cells per liter of medium: final reaction pH 7.3–7.5. Aliquots of the cultural medium were examined and no phenolic or carbonyl groups could be detected by the *p*-nitrosodimethylaniline (11) and 2,4-dinitrophenylhydrazine reagents. Cells grown in Medium 23YM oxidized 2,4-D, MCPA, 2,4,5-T, and 2,4-DCP (Fig. 6): ultraviolet absorption spectra of the metabolized substrates also indicated that they were degraded. The endogenous respiration was high but it could be reduced by using older cells (6–7 days) or by aeration of the resting cells prior to addition to the Warburg vessels. However, it was considered that unaerated cells which had just decomposed 2,4-D (Fig. 5) would serve best to give a preliminary indication of the capabilities of the organism. The data plotted in Fig. 6 indicate that Bacterium R oxidized the substrates as follows (the figures express percentages of complete oxidation to CO_2, H_2O, and HCl): 2,4-D, 82; MCPA, 67; 2,4,5-T, 33; and 2,4-DCP, 169. Data obtained from the respiration of these compounds must be interpreted with caution because Brody (15) found that 2,4-D stimulated the endogenous respiration of rat liver mitochondria and he suggested that it acted as an uncoupling agent. Likewise, since stimulation

of endogenous respiration by 2,4-dinitrophenol is commonplace, it is not surprising that 2,4-DCP seemed to behave similarly. Goldacre, Galston, and Weintraub (25) found that 10^{-7} to 10^{-5} *M* 2,4-DCP stimulated pea indoleacetic acid oxidase and the 2,4-DCP also might have behaved analogously in Bacterium R. The results of these workers emphasized the need for using herbicides of the highest possible purity and this was done in the work herein reported.

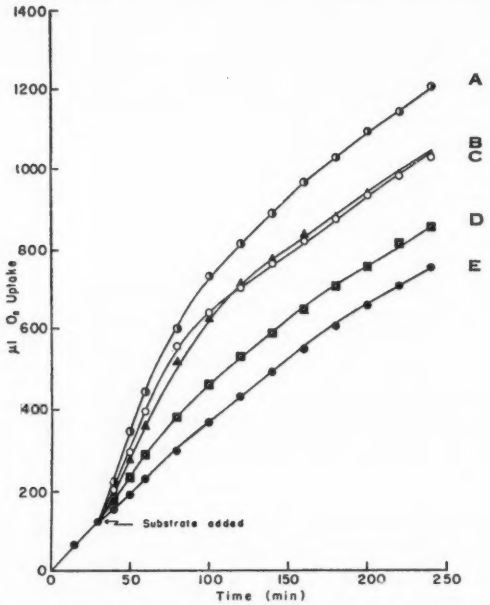

FIG. 6. Rate of oxygen uptake of resting cells of Bacterium R (E) following the addition of 2 micromoles of each of the following: 2,4-DCP (A), MCPA (B), 2,4-D (C), and 2,4,5-T (D). Cells grown 5 days at 28° C. in aerated Medium 23YM, harvested, washed once in distilled water, and stored overnight at 4° C.; washed twice next day and suspended in distilled water. Each of duplicate Warburg vessels contained 1 ml. of cell suspension equivalent to 23 mg. dry weight of cells; 1 ml. 0.066 *M* KH_2PO_4–Na_2HPO_4, pH 7; 1 ml. of substrate, pH 7; and 0.2 ml. of 3*N* KOH in the center well.

The organism behaved in an anomalous manner in that both citric acid and 0.1% yeast extract were inhibitory to growth on agar but stimulatory to growth in liquid media. Inhibition of growth on agar by citric acid might have been due to a combined chelating effect of both agar and citrate. Of particular interest is the fact that 2,4-DCP was found to accumulate when the cells were grown in Medium 23 containing 0.1% yeast extract. It has been established that this was not a photochemical reaction, that it was not due to lysis of the cells, and that no 2,4-DCP accumulated in the absence of 2,4-D. The 2,4-DCP was identified by two chromatographic techniques (42, 11). No 1,2-dihydroxyphenols (8), aldehydes, or ketones appeared in the medium. Replicate cultures differed both in the rate and final amount of 2,4-DCP

produced but of 13 cultures, 12 produced 2,4-DCP. It is probable that 2,4-DCP was responsible for the death of the bacteria in 1–2 weeks (See Table III) because the organism remained viable for at least 10 weeks when it was grown in media which did not cause the accumulation of 2,4-DCP. The 'blocking agent' in yeast extract which prevented the metabolism or caused the accumulation of 2,4-DCP has not been identified but it is ether-soluble, easily adsorbed on charcoal, stable to autoclaving at pH 7, and appeared to be neither thioctic acid nor a common amino acid.

The importance of the source of inoculum in governing the formation of 2,4-DCP is illustrated by the fact that inocula grown on Medium 23 plus fumarate formed 2,4-DCP in Medium 23 containing 0.1% yeast extract whereas inocula grown in Medium 23YM did not.

Release of Chloride from 2,4-D

Some indication of the degree of splitting of 2,4-D can be obtained by finding the amount of chloride released by cells decomposing the herbicide. The following experiment was devised to estimate the amount of chloride released by resting cells of Bacterium R in the presence of 2,4-D. Cells were grown for 6 days in 4 liters of aerated Medium 23YM following which they were harvested in a Sharples centrifuge, washed twice with distilled water, and stored in distilled water overnight at 4° C. The following day they were washed twice with distilled water, suspended in *ca.* 15 ml. of distilled water, and thoroughly mixed. 'Boiled cells' were prepared by heating an aliquot of the cell suspension in boiling water for 12 minutes. Cell suspensions were placed in 125 ml. Erlenmeyer flasks, the appropriate solutions added, and the flasks shaken at 25° C. At the end of 7 hours, the mixture was rinsed quantitatively into centrifuge tubes and the cells spun down at 18,000 *g*. After the supernatant was decanted into beakers, the cells were washed, spun down, and the washings added to the appropriate beaker. The solutions were titrated with silver nitrate using a Titrimeter (Fisher Scientific Co.). A mass of resting cells rather than a growing culture was used to estimate the release of chloride from 2,4-D because of the relative simplicity and accuracy involved but the results might differ according to the method used. The number of cells, length of incubation period, and concentration of 2,4-D ensured that all of the substrate was decomposed.

The results presented in Table V show that virtually all of the 2,4-D chlorine was released as chloride by the cells and also suggest that the release was enzymatic. Determination of the total endogenous halogen indicated that it could not have influenced the results by more than 1% even if it were all excreted by actively metabolizing cells. Rogoff and Reid (45) found a quantitative release of chloride from 2,4-D by growing cells of *Corynebacterium* sp. but Steenson and Walker (52) using growing cells of *Flavobacterium peregrinum* obtained only a 76% release of the 2,4-D chlorine after 39 days. Bray, Thorpe, and Vallance (14) using mammalian liver extracts found that chloride was liberated non-enzymatically from aliphatic chloro compounds

largely by an interaction of organic chlorine and SH compounds. No chloride was released from nuclear substituted aromatic compounds by the extracts (14).

TABLE V

RELEASE OF CHLORIDE FROM 2,4-D BY AERATED, RESTING CELLS

Flask	Constituents (ml.)					
	Cells		0.066 *M* phosphate buffer, pH 7	Na 2,4-D 5 μM./ml.	Water	Ml. 0.002 *N* $AgNO_3$
	Fresh	Boiled				
1,2	3		3	3		14.7
3,4	3		3		3	0.4
5		3	3	3		0.8
6		3	3		3	0.85
7			3	3	3	0.3

NOTE: Recovery, as chloride, of organic chlorine added = 94% (corrected for endogenous and reagent blank values).
ca. 70 mg. dry weight of cells per flask.

Discussion

Origin and Development of Cells

The selective development of bacteria capable of decomposing 2,4-D raises some interesting questions. Is the decomposition of 2,4-D the result of adaptive (inductive) or selective processes or a combination of both? It is improbable that bacteria in soil untreated with herbicide possess enzymes specific for the decomposition of 2,4-D but it is possible that cells having enzymes of low specificity, which will attack natural compounds structurally related to 2,4-D, may also attack 2,4-D. These cells would then selectively proliferate, without any qualitative change in their enzymic constitution. On the other hand, the persistence in soil of cells capable of decomposing 2,4-D (40) does not preclude the possibility that induced enzymes may be involved in the herbicide degradation because the active cells might be mutants capable of forming induced enzymes for the decomposition of 2,4-D. The over-all mechanism would amount to selection by ability to form induced enzymes. Some of the species reported in the literature to decompose 2,4-D may have arisen by one process, some by another. There is evidence (10) in the nature of a lag period in oxygen uptake when xylose-grown cells were exposed to 2,4-D that Bacterium R may have proliferated through the operation of induced enzymes. Steenson and Walker (52) concluded that one of their strains formed induced enzymes for the decomposition of MCPA because the cells, in order to be effective, had to be grown in the presence of the substrate; but it is possible that there was a selection of mutants. Furthermore, their data from resting cell experiments do not indicate the presence of induced enzymes. Audus (5) found that his organism became irreversibly 'de-adapted' to the attack of 2,4-D when grown in its absence and this behavior remains puzzling, unless it is assumed that soil components are necessary for the initiation of the adaptive or mutational process in this organism.

It can also be asked, do the cells arise from the indigenous (autochthonous) or zymogenous flora? Some evidence for the latter flora as a source of bacteria decomposing 2,4-D is that the herbicide remained for long periods in soil low in organic matter (39, 30, 16, 10) and that extracts of soil low in organic matter were relatively ineffective compared with extracts of soil high in organic matter in 'activating' sterile, high-organic-matter soil in percolators and Erlenmeyer flasks (10). It is possible that soil low in organic matter lacks only certain cofactors necessary for the proliferation of the effective bacteria. The activity of the cells in utilizing pentoses and urea also argues in favor of their origin from the zymogenous flora, but it must be pointed out that the organism can maintain itself on simple media.

Taxonomy of Bacterium R

Bacterium R can not be identified with any known species but it is allied most closely with the *Achromobacter*, according to the taxonomic systems of both Breed, Murray, and Hitchens (12) and Skerman (46). Corynebacteria have been implicated most frequently in the decomposition of 2,4-D and it is possible that Audus's (4) *B. globiforme* (*Arthrobacter globiforme*) is actually a *Corynebacterium* sp., particularly since it did not form cocci, one of the principal distinguishing characteristics of the *Arthrobacter*. Some confusion exists concerning Jensen and Petersen's (29) *Flavobacterium aquatile* because Weeks (54) found it to be a strain of *Sporocytophaga congregata* but their other 2,4-D decomposing isolate appeared to be a *Corynebacterium* species. Nevertheless, some members of the Achromobacteriaceae are also important 2,4-D decomposers. Stapp and Spicher (51) isolated a new *Flavobacterium* and Steenson and Walker (52) a new *Achromobacter* species capable of degrading 2,4-D. It is surprising that no pseudomonads or vibrio have been found to decompose 2,4-D in view of their versatility in decomposing other aromatic compounds (50, 19).

It would perhaps be advisable if the criteria of "peritrichous or nonmotile" (12) for the identification of Achromobacteriaceae were broadened to include other forms of flagellation because the description of many species was made when diagnostic and cultural methods were inadequate. Skerman's (47) suggestion that "consideration be given to a complete re-examination of all recognized species along certain specified lines" might also be appropriate. The variety of flagellar types occurring in cultures of Bacterium R and other organisms described in the literature (26, 32) makes the type of flagellation of limited value for diagnostic purposes.

Nutritional Characteristics

In order to serve as the sole source of carbon, 2,4-D must be transformed into all of the key intermediates necessary for the synthesis of cell protoplasm. In view of this somewhat rigorous requirement it is not surprising that the growth of Bacterium R was stimulated by a number of substances, the most effective of which were the dicarboxylic acids of the tricarboxylic acid cycle. Bernheim (13) has used the oxidation of succinate by resting cells of *P.*

aeruginosa in the presence of ammonium ions to increase the synthesis of induced enzyme precursors for benzoate oxidation. It is possible that the dicarboxylic acids acted in this manner by aiding ammonia assimilation and protein (enzyme) synthesis, the process being in part autocatalytic. Perhaps, too, the stimulatory acids 'sparked' the oxidation of toxic compounds which might otherwise have accumulated. The acids also might have been utilized as C_1 units, in view of the demonstrated ability of the cells to utilize formate, bicarbonate, and urea.

Bacterium R probably differs little from the parent soil type because it has retained its original morphological and biochemical characteristics despite artificial cultivation.

Bacterial Degradation of 2,4-D

Now that useful quantities of consistently active cells can be grown with ease in reproducible media, work is in progress to elucidate the mechanism of decomposition of 2,4-D but the present investigation provides some information regarding the over-all process. Ordinarily, no phenols, aldehydes, or ketones accumulated when 2,4-D was metabolized by either resting or growing cells. Moreover, the high degree of oxidation of 2,4-D by resting cells, coupled with the quantitative release of chloride from the herbicide further suggests that 2,4-D was decomposed to small units. The formation of 2,4-DCP by cells grown in the presence of 0.1% yeast extract and 2,4-D might have been due to components of the extract causing the dissociation of the culture to forms which could not decompose 2,4-D beyond 2,4-DCP but it is also possible that compounds in the yeast extract caused a blockage of the 2,4-DCP oxidizing system. There is insufficient evidence to establish whether 2,4-DCP is a 'normal' or an abnormal' metabolite of 2,4-D in this organism.

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EFFECT OF ANTIBIOTICS ON OXIDATION OF PROGESTERONE BY TWO STREPTOMYCETES[1]

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Abstract

Studies of the microbial conversion of progesterone to $\Delta^{1,4}$-androstadiene-3,17-dione by *Streptomyces lavendulae* and to 16α-hydroxyprogesterone by *Streptomyces* sp. ATCC 11,009 have shown that a lag in metabolism of the steroid occurs when the progesterone is added to washed cells of these organisms grown in a medium containing soybean meal, glucose, and calcium carbonate. No lag was observed when the cells were grown in this medium supplemented with a trace of progesterone. Addition to the cell suspensions of sufficient quantities of streptomycin sulphate, viomycin sulphate, dihydrostreptomycin sulphate, neomycin B sulphate, or tetracycline hydrochloride (to give a concentration of 10 μg. per ml.) coincident with the addition of the progesterone resulted in complete inhibition of steroid oxidation. When addition of the antibiotics was delayed until 12 hours after addition of the progesterone or the cells were grown in a medium containing a trace of progesterone, practically no inhibition was observed. These experiments suggest that the enzymes formed in these two streptomycetes which carry out the transformation of progesterone are adaptive in origin.

In previous publications from these laboratories we have described the conversion of progesterone (Δ^4-pregnene-3,20-dione) to 16α-hydroxyprogesterone (Δ^4-pregnene-16α-ol-3,20-dione) by an unidentified streptomycete (Perlman *et al.* (8, 9, 10)) and to $\Delta^{1,4}$-androstadiene-3,17-dione and Δ^1-testosterone ($\Delta^{1,4}$-androstadiene-17β-ol-3-one) by *Streptomyces lavendulae* (Fried *et al.* (4, 5)). In further study of these microbial transformations we have found that a number of antibacterial antibiotics inhibit the formation of enzymes involved in the oxidation of progesterone by these streptomycetes.

Methods

Cultures of the two streptomycetes, *S. lavendulae* (ATCC 8664) and *Streptomyces* sp. ATCC 11,009, were maintained on agar slants as well as in lyophilized form as previously described (Perlman *et al.* (10)). The streptomycetes were grown for these experiments in cotton-plugged 250 ml. Erlenmeyer flasks containing 50 ml. of medium. After inoculation the flasks were placed on a rotary shaker (280 r.p.m.; 2 in. amplitude) located in a room maintained at 25° C. The medium used contained 15 g. extraction process soybean meal, 20 g. of glucose, 5 g. of calcium carbonate (sterilized separately as a dry powder), and 1 liter of distilled water. Inoculum was prepared by adding a quantity of a spore suspension or vegetative cells (previously grown on this medium) to flasks of this medium and then placing the flasks on the rotary shaker. After 2 days' incubation, 5 ml. of the suspension was transferred to a second flask and incubation continued for a second 2 day period. Five-milliliter portions of the cellular suspension formed during this period were then used to inoculate a number of flasks of the soybean meal – glucose – calcium carbonate medium and these flasks were placed on

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the shaker. After 2 days' incubation the contents of a number of replicate flasks were pooled and the solids collected by centrifugation. The collected material was resuspended in sterile distilled water to the original volume and shaken for a few minutes on a mechanical shaker. The solids were again collected by centrifugation and resuspended. Ten-milliliter aliquots of this washed-cell suspension were then added to 250-ml. Erlenmeyer flasks containing 40 ml. of sterile distilled water. A 0.125 ml. aliquot of an acetone solution of progesterone (1 g. of steroid per 5 ml. of acetone) was then added to each flask (resulting in a concentration of 0.5 mg. of steroid per ml. of cell suspension) and the flasks placed on the rotary shaker. The pH of these suspensions ranged from 5.9 to 6.4. Macroscopic observation of the suspensions showed the presence of only a few undigested particles of soybean meal.

After the incubation periods indicated in Figs. 1 and 2 and Table I, aliquots of the cell suspension were removed for analysis of the steroid components. Usually 10 ml. portions of the sample were extracted with 5 ml. of the methyl-*iso*-butyl ketone by vigorous shaking on a mechanical reciprocating shaker. Aliquots of the extract from the *S. lavendulae* fermentations were analyzed for residual progesterone, $\Delta^{1,4}$-androstadiene-3,17-dione, and Δ^{1}-testosterone by a semiquantitative modification of the filter paper chromatographic method described by Burton *et al.* (2) using diethyleneglycol mono ethylether as the stationary phase and methylcyclohexane as the moving phase in the chromatographic system. A fluorescent scanner (Haines (6)) was used to locate the steroids on the papergram. An estimate of the quantity of steroid present in each spot was made by eluting the steroid-containing area with a known volume of 95% ethanol and measuring the optical density in a Beckmann spectrophotometer (model DU) at 240 mμ. Fermentation samples containing 16α-hydroxyprogesterone were tested in a similar manner except that the filter paper chromatograms were developed with a solvent system in which propylene glycol was the stationary phase and toluene the moving phase (Burton *et al.* (2)). In all the filter paper chromatographic analyses aliquots of solutions containing progesterone, $\Delta^{1,4}$-androstadiene-3,17-dione, Δ^{1}-testosterone, and 16α-hydroxyprogesterone were placed on the filter paper sheets to aid in identification of the steroids in the extracts and to assist in quantitative analysis. The recovery of steroids in these filter paper chromatographic analyses ranged from 90 to 112% of that added, and, therefore, it was felt that the values obtained in analyses of extracts of fermentation samples were satisfactory for comparative purposes.

Results

Analysis of samples removed periodically from the suspensions of *S. lavendulae* with progesterone over a 36 hour period showed that a rather long delay occurred before appreciable quantities of the steroid were oxidized. Nearly all of the metabolized progesterone was transformed to $\Delta^{1,4}$-androstadiene-3,17-dione and only traces of Δ^{1}-testosterone and other steroidal metabolites were found in the extracts of the fermentation samples. Some of the data

collected in these experiments are summarized in Fig. 1 (*a*). When the *S. lavendulae* cells grown on a soybean meal – glucose – calcium carbonate medium supplemented with 0.05 g. per liter of progesterone were used in a similar experiment, the results summarized in Fig. 1 (*b*) were obtained. As the lag period was not found in the experiments with cells grown in media containing the progesterone, these data suggest the adaptive nature of the enzymes in *S. lavendulae*. Results obtained in similar experiments on the conversion of progesterone to 16α-hydroxyprogesterone by *Streptomyces* sp. ATCC 11,009 are summarized in Fig. 2. It is evident from this study that the enzymes involved in oxidizing progesterone in this streptomycete, too, may be adaptive in origin.

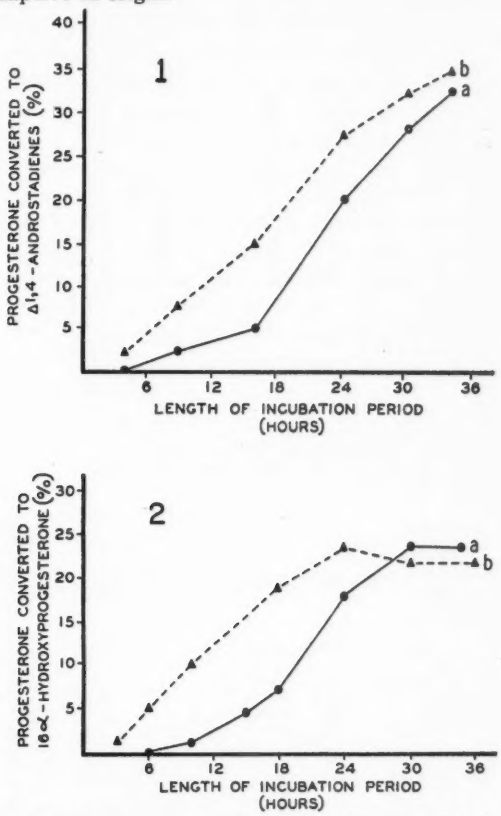

FIG. 1. Conversion of progesterone to $\Delta^{1,4}$-androstadienes by washed cells of *Streptomyces lavendulae*.
a—Cells grown without progesterone.
b—Cells grown in media containing progesterone.

FIG. 2. Conversion of progesterone to 16α-hydroxyprogesterone by washed cells of *Streptomyces* sp. ATCC 11,009.
a—Cells grown without progesterone.
b—Cells grown in media containing progesterone.

TABLE I

EFFECT OF ANTIBIOTICS ON CONVERSION OF PROGESTERONE TO $\Delta^{1,4}$-ANDROSTADIENES BY *Streptomyces lavendulae*

Antibiotic added	Concn., μg./ml.	Effect on conversion, % inhibition: Condition A	Condition B	Antibiotic added	Concn., μg./ml.	Effect on conversion, % inhibition: Condition A	Condition B
Streptomycin sulphate	30	>95	< 5	Sodium benzylpenicillinate	200	75	5
	10	90	5		100	15	5
Dihydrostreptomycin sulphate	30	95	< 5	Erythromycin	200	95	5
	10	95	5		100	45	5
Neomycin B sulphate	30	>95	< 5	Polymyxin B sulphate	200	90	5
	10	95	5		100	35	5
Viomycin sulphate	30	>95	< 5	Subtilin	200	85	<5
	10	95	5		100	25	5
Tetracycline hydrochloride	30	>95	< 5	Tyrothricin	200	90	<5
	10	95	5		100	35	<5
Methymycin	200	95	5	Gramicidin	200	90	<5
	100	55	10		100	25	<5
				Bacitracin	100	25	<5

A—Cells grown without progesterone in medium.
B—Cells grown with progesterone in medium.

Procedure: Culture grown for 2 days on soybean meal – glucose – $CaCO_3$ medium (see text). Cells collected by centrifugation and resuspended in sterile distilled water (1 volume of cells to 9 volumes of water). 20 ml. aliquots added to 125 ml. Erlenmeyer flasks. Progesterone dissolved in acetone (1 g. in 5 ml.) added to give concentration of 500 μg. per ml. Antibiotics added to give indicated concentration. Mixture shaken for 24 hours at 25° C. 21% of progesterone in unsupplemented control flasks A converted to $\Delta^{1,4}$-androstadienes as determined by filter paper chromatography. Conversion in control flasks B was 28% of progesterone added.

Data collected in experiments where a number of antibiotics were added to the *S. lavendulae*–progesterone mixture are summarized in Table I. Addition of streptomycin sulphate, dihydrostreptomycin sulphate, viomycin sulphate, neomycin B sulphate, or tetracycline hydrochloride to give concentrations of the order of 10 μg. per ml. almost inhibited the metabolism of progesterone by these cells when the antibiotics were added to the cell suspension coincident with the addition of the progesterone. In a number of experiments it was found that addition of any one of the foregoing antibiotics to the cells 12 hours after addition of the progesterone did not affect the metabolism of the steroid. We also observed that if the cells were grown on progesterone-containing media, these antibiotics had no effect even if the concentration was raised to 200μg. per ml. As shown in Table I addition of gramicidin, tyrothricin, subtilin, bacitracin, polymyxin B sulphate, erythromycin, methymycin, or sodium benzylpenicillinate at concentrations of the order of 100 μg. per ml. to the cell suspension coincident with the addition of the progesterone inhibited the metabolism of the progesterone significantly and at levels of 200 μg. per ml. completely inhibited steroid metabolism. In these experiments, in which the effect of addition of these antibiotics to the washed cell-progesterone suspension was studied, the progesterone was metabolized to $\Delta^{1,4}$-androstadiene-3,17-dione and only traces of other steroid metabolites were found. Antibiotics not inhibitory at levels of 1 mg. per ml. when added to the cell suspension coincident with the addition of progesterone included cycloheximide, thiolutin, amphotericin A, rimocidin, nystatin, and fumagillin.

A less extensive series of experiments using *Streptomyces* sp. ATCC 11,009 are summarized in Table II. These data show that addition of streptomycin sulphate, neomycin B sulphate, or dihydrostreptomycin sulphate to washed cell suspensions to give a concentration of 10 μg. per ml. resulted in almost complete inhibition of transformation of progesterone when the antibiotic was added to the cells coincident with the addition of the progesterone.

TABLE II

EFFECT OF ANTIBIOTICS ON CONVERSION OF PROGESTERONE TO 16α-HYDROXYPROGESTERONE BY *Streptomyces* SP. ATCC 11,009

Antibiotic added	Concn., μg./ml.	Effect on conversion, % inhibition	
		Condition A	Condition B
Streptomycin sulphate	30	>90	< 5
	10	90	5
Neomycin B sulphate	30	>90	< 5
	10	90	5
Dihydrostreptomycin sulphate	30	>90	< 5
	10	90	5

A—Cells grown without progesterone in medium.
B—Cells grown with progesterone in medium.

Procedure: As in Table I. 23% of progesterone in control flask A was converted to 16α-hydroxyprogesterone as determined by filter paper chromatography. Conversion in control flask B was 27% of the progesterone added.

These antibiotics had no effect on the metabolism of the added progesterone if the cells were grown in media containing a small quantity of progesterone or if they were added to the cell-suspension 12 hours or more after the addition of the progesterone.

Discussion

The above experiments suggest that the enzymes formed in these two streptomycetes which carry out the transformation of progesterone are adaptive in origin. This is similar to the conclusion reached by Rubin *et al.* (12) regarding the nature of the steroid hydroxylating enzymes of *Aspergillus niger*, and in agreement with the theory of Marcus and Talalay (7) concerning the origin of the α and β hydroxysteroid dehydrogenases in *Pseudomonas testosteroni*.

The inhibition of the formation of the steroid-oxidizing system by addition of streptomycin, neomycin B, and other antibiotics is perhaps further evidence of the adaptive nature of the enzyme system. This effect of antibiotics on formation of enzymes in microorganisms has been reported by Creaser (3) and Polglase (11), who have shown that formation of the adaptive enzyme β-galactosidase in *Staphylococcus aureus* and in *Escherichia coli* is inhibited by antibiotic concentrations which do not inhibit cell growth. Arima *et al.* (1), in a similar study of the effect of streptomycin on formation of *m*-hydroxybenzoic acid oxidase in *Pseudomonas ovalis*, found that addition of streptomycin at the start of the enzyme induction period inhibited formation of the enzyme. However, once initiation of adaptive enzyme formation had begun, addition of streptomycin did not inhibit further enzyme formation.

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COUMARINS AND PHENOLIC ACIDS IN THE UREDOSPORES OF WHEAT STEM RUST[1]

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Abstract

A paper chromatographic method suitable for identification of the small amounts of coumarins and phenolic acids present in the uredospores of wheat stem rust was developed. By the use of the circular technique and a combination of three different solvent systems an adequate separation of all the substances was achieved. A preliminary development of the chromatogram with a solvent in which the test compounds were non-mobile facilitated identification and avoided the need for extensive preliminary fractionation of the extracts.

Using this method the following compounds were identified in spore extracts: coumarin, umbelliferone, daphnetin, aesculetin, *p*-hydroxybenzoic acid, vanillic acid, protocatechuic acid, *o*-coumaric acid, *p*-coumaric acid, ferulic acid, and caffeic acid; coumarin, *p*-hydroxybenzoic acid, vanillic acid, protocatechuic acid, *o*-coumaric acid, and ferulic acid were also present as glycosides; in addition scopoletin, gallic acid, syringic acid, and sinapic acid were detected after hydrolysis and are assumed to be present only in a bound form.

In order to obtain some information about the role of these substances in the physiology of wheat stem rust, uredospores were germinated by being floated en masse on dilute aqueous solutions. Of the compounds tested, indoleacetic acid, coumarin, *o*-coumaric acid, protocatechuic acid, umbelliferone, and daphnetin gave a marked stimulation of germination at concentrations of 10–200 μg./ml. Caffeic acid, vanillic acid, *p*-hydroxybenzoic acid, ferulic acid, and ferulic acid β-glucoside had little effect or were strongly inhibitory.

The stimulation of germination is attributed to the counteraction of a self-inhibitor released from the spores, and the possible significance of the compounds on the physiology of the rust and the host–parasite relationship is discussed.

Introduction

Erratic germination of the uredospores of *Puccinia graminis* var. *tritici* is a common phenomenon and recent observations have suggested that the formation of germination inhibitors by the spores themselves is largely responsible. Allen (2) has shown that heavy concentrations of old spores floated aerobically on water or acidic buffers do not germinate. Transferring the spores to fresh water, or the use initially of very low concentrations of spores resulted in rapid germination and it was considered that the inhibition was caused by a water soluble metabolite released from the spores. The presence of such an inhibitor in the medium on which the spores had been floated en masse was confirmed, and evidence was obtained that a volatile acid might be responsible for most of the activity. The importance of a volatile inhibitor released by the spores was also stressed by Forsythe (14), who considered that the substance might be 2-methylbutene-2. Yarwood

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(42), using as evidence the effect on germ tube length of increasing spore density, suggested that the spores of *Uromyces phaseoli* contained a factor causing stimulation of germination, as well as a germination inhibiting factor. The concentration of these substances in the agar medium on which the spores rested appeared to be proportional to the number of spores used per unit volume. Cross protection between the rusts of the bean and sunflower has also been demonstrated by Yarwood (41), the implication suggested being that release of inhibitory substances by *Uromyces phaseoli* protects the sunflower leaves from subsequent attack by their natural parasite *Puccinia helianthi*. In a similar manner sunflower rust spores protect the bean plant from infection by *Uromyces phaseoli*. In the transfer of the inhibitor from the rust to the plant, it was suggested that the water solubility of the factor was of greater importance than its volatility.

Although self-inhibition of germination has only infrequently been reported in fungal spores, its occurrence in the seeds of higher plants is widespread (13, 16). In spite of this, only in comparatively few instances have the active substances been identified. In common with the inhibitory substances in rust spores, many of these are stable compounds of low molecular weight, readily leached from the seed with water (5, 7, 10, 12, 32, 33, 38). The well known action of coumarin on seed germination and its possible role in dormancy (27) led us to investigate the presence of this and related phenolic compounds in the spores of *Puccinia graminis* var. *tritici*.

Although the paper chromatographic separation of such compounds has already been reported by previous investigators (4, 9, 36, 40), none of the methods described proved satisfactory for their identification in rust spore extracts. Because of the very small amounts of these substances present and the difficulty of separating them by preliminary fractionation procedures from contaminating material, particularly ether soluble acidic pigments and lipoidal substances, paper strip chromatograms invariably gave severe streaking with the resultant uncertainty about R_f values and obscuring of fluorescent, absorbing, and colored zones present in the extracts. The use of the circular paper chromatographic technique overcame this difficulty. The heavy applications of spore extracts necessary to give adequate concentrations for the detection of minor components in the mixture resulted in distortion of the solvent front, particularly serious in the aqueous solvent mixtures which have been commonly found to give the best separations of these types of compounds. In extreme cases the applied sample impeded the solvent flow completely in its sector of the chromatogram.

The use of non-polar solvents as the mobile phase was considered desirable but those tried, e.g. the toluene – acetic acid – water mixture recommended by Bate-Smith (4), gave either very diffuse zones or poor separation owing to unsuitable R_f values. In acidic systems, these tended to be too high, whereas in alkaline systems the majority of the compounds of interest did not move from the origin. Satisfactory separations of the compounds as the free acids were, however, obtained when the paper was impregnated with formamide

and non-polar solvents used as mobile phase (8). The use of solvent mixtures containing a considerable proportion of water was found to be possible if the chromatogram was given a preliminary development under alkaline conditions with a non-polar solvent (e.g. benzene, petrol) to move lipoid substances to the outer edge of the paper. For the complete separation of all the compounds tested, a combination of three different solvent systems was finally chosen. The speed, simplicity, and excellent resolution obtained by the circular method permit rapid separation and identification of this group of compounds in natural extracts. Moreover, since known substances suspected to be present in the extract can be run in adjoining, and if desirable, even overlapping segments of the chromatogram, identification of unknowns by comparison of R_f values and color reactions is facilitated.

Materials and Methods

Solvent Systems

System A.—The paper was first dipped in a mixture of commercial formamide and ethanol (1:1), well blotted between sheets of fresh filter paper, and the samples applied. After being allowed to stand for about 15 minutes for evaporation of the ethanol, the paper was transferred to the chromatography tank and irrigation was begun with a mobile phase consisting of a mixture of benzene and petrol (b.p. 100°–120° C.) in the proportions 1:2.

System B.—The procedure was the same as for A except that chloroform saturated with commercial formamide was used as mobile phase.

System C.—The paper was first buffered by being dipped in a solution of 1 *M* potassium phosphate of pH 7.4, blotted, and allowed to dry before the samples were applied. The mobile phase was a mixture of *sec*-butanol and water in the proportion 4:1 (v/v).

Procedure

Samples were applied from a capillary to segments of a small circle drawn around the center of a 32 cm. disc of Whatman No. 1 filter paper. The chromatography tank consisted of a large desiccator (24 cm. internal diameter), the filter paper disc being placed on the upper rim and covered with the lid. Solvent was fed to the center of the paper by means of a thin cotton wick from a reservoir supported within the desiccator. As far as possible the system was allowed to equilibrate with the volatile solvent mixture before use, but with the simple equipment described a completely equilibrated system is impossible. Chromatograms were run in a constant temperature room at 24° C.

The filter paper was normally removed from the desiccator when the solvent front had reached the outer edge of the desiccator rim. However, in those instances where it was desirable to obtain better separation of very slow moving components, and fast moving components were absent or of no interest, the chromatogram was allowed to develop for longer periods of time. Evaporation of the mobile phase from the strip of paper outside the desiccator rim allows a

further steady flow of solvent. The position of many of the fluorescent zones could be viewed through the desiccator lid with a lamp of 3660 Å and these were conveniently used as markers.

Where heavy concentrations of spore extract were applied, the chromatogram was given a preliminary development with petrol (b.p. 100°–120° C.) as mobile phase to move lipoid impurities out from the origin. After the solvent had reached the outer edge of the desiccator rim the paper was removed, dried in the air, and reinserted in a second desiccator with the appropriate solvent system.

Detection of Zones

Coumarins and phenolic compounds were detected by the methods described by Swain (36). Examination of the chromatogram under ultraviolet light (2537 Å) for absorbing and fluorescent zones, both before and after spraying with 1 *N* sodium hydroxide solution, was followed by development with a chromogenic spray consisting of diazotized *p*-nitroaniline in acetate buffer. Since the chromatogram was frequently sprayed only lightly with sodium hydroxide solution and then heavily with the acetate buffer, a final spray with 5% Na_2CO_3 was normally used to return the paper to a strongly alkaline condition.

Origin of Spores

The uredospores used in this investigation were gathered late in August 1956 with a cyclone collector from a plot of Stewart wheat kindly provided by Dr. D. R. Knott of the Field Husbandry Department, University of Saskatchewan. This plot had been inoculated earlier in the summer with spores of race 15B stem rust. Because of the host resistance to leaf rust as well as other races of stem rust, mixing of races was kept to a minimum. The spores collected were stored in sealed glass jars in 50-g. lots at 5° C. until used 2 months later. These spores were not used in the germination tests. For this purpose freshly collected mature spores of the same race were obtained from infected Stewart wheat growing in a light chamber.

Extraction of Spores

Four-gram samples of the spores were extracted in a Soxhlet apparatus with diethyl ether for 2 hours. The ether extract was evaporated to dryness and distributed in a mixture of 50 ml. of methanol and 50 ml. of petrol (b.p. 30°–60° C.). The supernatant phase containing the bulk of the lipid material was separated and discarded; the remaining solution was concentrated as above to a small volume (Extract 1A). The residual spore material was dried and extracted by being shaken at room temperature with water (150 ml.) for 4 hours. The aqueous extract was separated by filtration, acidified with dilute hydrochloric acid to pH 4.0, and thoroughly extracted with diethyl ether. The ether extract was concentrated to a small volume (Extract 1B). A second extraction of the aqueous solution with ether was carried out to ensure that all ether extractable material had been removed.

Paper chromatographic examination of this extract showed no coumarins or phenolic compounds present. The aqueous phase was then freed of ether by evaporation at 40° C. for 10 minutes *in vacuo*, and incubated with emulsin at 40° C. for 3 hours. A further ether extraction was then carried out and the extract concentrated (Extract 1C).

A larger quantity (27 g.) of spores of the same age was examined; the rust oil fraction had been removed for a separate investigation by grinding these spores in a ball mill for 5 days with diethyl ether. The residual spore material was extracted in a Soxhlet apparatus for 24 hours with methanol. The extract was evaporated to dryness at 40° C. *in vacuo* and redissolved in 200 ml. of water. The aqueous solution, adjusted to pH 3.5, was then continuously extracted with ether for 24 hours, and the ether extract concentrated (Extract 2A). A further extraction for 4 hours with ether was carried out to ascertain that all ether soluble materials had been removed. The remaining aqueous solution was adjusted with concentrated hydrochloric acid to approximately 1 *N* and heated on a steam bath for 6 hours. A final ether extraction was made in a continuous extractor for 24 hours, and the extract again concentrated (Extract 2B). Except where otherwise stated, all concentrations were carried out in open vessels at room temperature; a gentle stream of nitrogen directed at the surface of the liquid was often used to speed the evaporation of the solvent.

Paper Chromatographic Identification

Extracts were chromatographed using the circular technique and the solvent systems described above. Identification of unknown substances in the extracts was established by comparing, in every case, the R_f value, the behavior under ultraviolet and visible light, and the color produced by the chromogenic spray reagent with those of known substances with which they were suspected to be identical.

Germination Tests

The substances tested for their effects on germination were dissolved in water at a concentration of 1000 μg./ml. and suitable dilutions were made. Poor solubility of some compounds at this level was overcome by warming and vigorous shaking. For ferulic acid-β-glucoside it was necessary to adjust the pH with alkali to effect solution.

Germination tests were made by spreading 1 mg. of freshly collected uredospores evenly over the surface of 0.3 ml. test solution contained in the 1.5 cm. diameter wells of plate glass slides. The slides were placed in a moist chamber and held at room temperature in front of a north window. A rapid preliminary survey was made by counting the number of germinated spores present after 2 to 3 hours in three low power microscope fields selected at random on the water surface and averaging the results. Minor variations in the controls from day to day were eliminated by setting all controls to a constant level and adjusting the other figures accordingly. It was noticed that the visual macroscopic color change in the spore layer after approximately

24 hours also gave quite a clear picture of the inhibitory or stimulatory properties of the various compounds. Therefore a further series of tests was made leaving the mounts undisturbed for 24 hours at which time they were photographed against a black background (Fig. 2). Then a segment of the surface spore film from each slide was removed and mounted in acid fuchsin – lactophenol. Finally counts were made on several hundred spores on each slide to determine the percentage germination which had taken place.

Results

Paper Chromatography

Only coumarin among the compounds tested was found to move out from the origin in solvent system A, and gave an R_f value of 0.52. A summary of R_f values, behavior in ultraviolet light, and color reactions with the chromogenic spray of compounds in the other two solvent systems used is given in Tables I and II. In each case coumarin moved at the solvent front.

The use of the formamide–chloroform system (B) allowed the identification of the coumarin derivatives, which moved somewhat faster than the phenolic acids. The faster running members of the latter group were also well separated, but the more heavily substituted compounds such as caffeic, chlorogenic, gallic, and protocatechuic acids remained at or close to the origin

TABLE I

R_f VALUES OF COUMARINS, BENZOIC ACID, AND CINNAMIC ACID DERIVATIVES

Substance	Solvent system	
	$CHCl_3$–formamide	*sec*-BuOH–phosphate buffer pH 7.4 (4: 1)
Coumarin	1.0	1.0
Umbelliferone	0.43	0.97
Herniarin	0.03	0.83
Scopoletin	0.67	0.91
Aesculetin	0.06	0.65
Daphnetin	0.10	0.68
4-Hydroxycoumarin	0.18	0.63
4-7-Dihydroxycoumarin	0.02	0.35
7-Methoxy-4-hydroxycoumarin	0.22	0.78
Dicoumarol	0.33	1.0
p-Hydroxybenzoic acid	0.04	0.37
Vanillic acid	0.16	0.27
Protocatechuic acid	0.0	0.12
Salicylic acid	0.13	0.70
Veratric acid	0.36	0.44
Gallic acid	0.0	0.05
Trimethylgallic acid	0.38	0.57
Cinnamic acid	0.35	0.81
o-Coumaric acid	0.09	0.51
p-Coumaric acid	0.06	0.44
Ferulic acid	0.09, 0.22	0.32
Caffeic acid	0.0	0.17
Chlorogenic acid	0.0	0.15
Syringic acid	0.25	0.21
Sinapic acid	0.13, 0.28	0.24

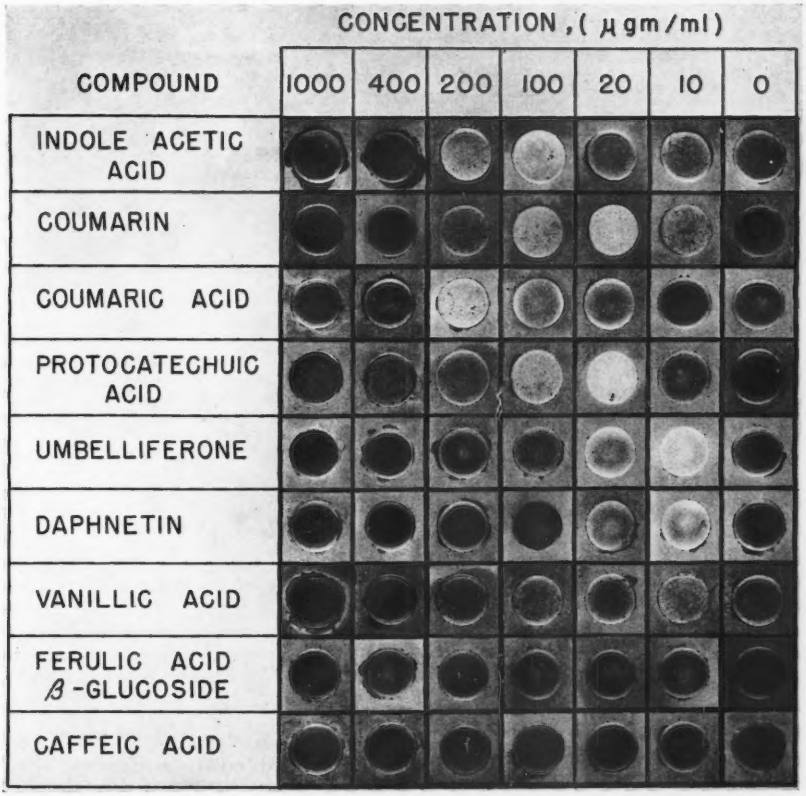


FIG. 2. Effect of various compounds on germination of uredospores.

TABLE II

APPEARANCE IN ULTRAVIOLET LIGHT AND AFTER SPRAYING WITH DIAZOTIZED *p*-NITROANILINE

Substance	Ultraviolet light		Diazotized *p*-nitroaniline
	Untreated	After NaOH	
Coumarin	Absorbing	Bright yellow fluoresc.	Violet
Umbelliferone	Bright blue fluoresc.	Bright blue fluoresc.	Brown
Herniarin	Violet fluoresc.	Blue fluoresc.	Violet-blue
Scopoletin	Bright blue fluoresc.	Blue-green fluoresc.	Gray-green
Aesculetin	Blue-white fluoresc.	Yellow fluoresc.*	Brown
Daphnetin	Faint yellow fluoresc.	Brown absorbing*	Brown
4-Hydroxycoumarin	Faint blue fluoresc.	Faint blue fluoresc.	Yellow
4-7-Dihydroxy-coumarin	Bright green-white fluoresc.	Bright blue-white fluoresc.	Orange-red
7-Methoxy-4-hydroxycoumarin	Faint blue fluoresc.	Faint blue fluoresc.	Yellow-brown
Dicoumarol	Absorbing	Absorbing	Yellow
p-Hydroxybenzoic acid	Absorbing	Absorbing	Red
Vanillic acid	Absorbing	Absorbing	Violet-blue
Protocatechuic acid	Faint blue fluoresc.	Absorbing‡	Violet-brown
Salicylic acid	Blue fluoresc.	Blue fluoresc.	Orange-brown
Veratric acid	Faint blue fluoresc.	Absorbing	—
Gallic acid	Faint blue fluoresc.	Dark brown absorbing‡	Dark brown
Trimethylgallic acid	Absorbing	Absorbing	—
Cinnamic acid	Absorbing	Absorbing	—
o-Coumaric acid	Yellow fluoresc.	Bright yellow fluoresc.	Violet
p-Coumaric acid	Absorbing	Light blue fluoresc.	Deep blue
Ferulic acid	Green fluoresc.	Bright green fluoresc.	Green
Caffeic acid	Blue fluoresc.	Orange-yellow fluoresc.*	Yellow-brown
Chlorogenic acid	Green-white fluoresc.	Yellow-green fluoresc.*	Brown
Syringic acid	Dark blue fluoresc.	Dark blue fluoresc.	Dark blue
Sinapic acid	Green fluoresc.	Green fluoresc.†	Green

*Strong yellow in visible light.
†Faint yellow in visible light.
‡Brown in visible light.

and were not resolved. System C was used to obtain a separation of these compounds, but proved to be unsuitable for the identification of the faster running coumarin derivatives, which moved at or near the solvent front.

The use of a basic as well as an acidic solvent system was advantageous since the R_f values of many of the compounds, relative to each other, changed considerably when they were chromatographed in the ionized form. A solvent system consisting of *sec*-butanol – concentrated ammonia (4: 1) was also found to give good separation, with R_f values considerably slower than in system C, and similar to B. The relative position of the compounds was as expected, similar to that found in C.

In general, with alkaline systems such as C, increasing the proportion of water in the solvent mixture increased the R_f values, while at the same time increasing the resolution of the zones. With a constant ratio of water to alcohol the R_f value decreased with increasing molecular weight of the alcohol used. A wide control of the separations achieved is therefore possible. In practice it was found that mixtures containing less than 20% water gave zones

too diffuse for good resolution, and in order to obtain suitably low R_f values for the substances examined it was desirable to use one of the butyl alcohols.

Double zones were observed in the non-aqueous solvent system formamide–chloroform for ferulic and sinapic acids, and other cinnamic acid derivatives gave noticeably broader zones than did the derivatives of benzoic acid. Presumably this is due to the separation of *cis*- and *trans*-isomers which has been noted previously by Williams (40) to occur in predominantly aqueous solvent mixtures. No separation of isomers was observed in the aqueous butanol system, in agreement with Williams' observations.

Identification of Compounds

A summary of the paper chromatographic investigation of the various spore extracts is given in Table III. It can be seen that a considerable number of phenolic acids as well as coumarins are present in the free state in the spores. Only coumarin is removed completely by thorough extraction with diethyl ether, the other substances being best obtained by treatment with methanol. Although no attempt was made to obtain quantitative estimations, protocatechuic acid appeared to be present in greatest amount. *p*-Hydroxybenzoic acid and umbelliferone also gave stronger zones than the remaining substances.

Since ether extracts immediately prior to hydrolysis were devoid of these compounds, it is assumed that the substances found in extracts 1C and 2B are present in the spores in a combined form, probably as glycosides since they

TABLE III

SUBSTANCES DETECTED IN EXTRACTS OF UREDOSPORES OF WHEAT STEM RUST

	Extract*				
	1A	1B	1C	2A	2B
Coumarin	++	+	+		
Umbelliferone	+	+		++	
Scopoletin			+		++
Daphnetin				+	
Aesculetin				+	
p-Hydroxybenzoic acid	+	+	+	++	+
Vanillic acid	+	+	+	+	++
Protocatechuic acid	+	+		+++	+
Gallic acid					+
o-Coumaric acid	+	+	+	+	
p-Coumaric acid				+	
Ferulic acid	+		+	+	++
Caffeic acid	+	+		+	
Syringic acid					+
Sinapic acid					+

*1A, direct ether extract of spores.
1B, ether extract of subsequent aqueous extract.
1C, ether extract after enzymatic hydrolysis of glycosidic fraction.
2A, ether extract of material removed from spores with methanol (large scale).
2B, ether extract after acid hydrolysis of glycosidic fraction (large sale).
Relative amounts of compounds in the extracts indicated approximately by multiple symbols.

are liberated by the action of β-glucosidase as well as dilute hydrochloric acid. Vanillic acid and scopoletin were found in strongest concentration in this fraction.

It is of some interest that in the three classes of compounds identified, coumarins, benzoic acids, and cinnamic acids, a parallel pattern of hydroxylation and methoxylation was found in almost all cases (Fig. 1). For example *p*-hydroxybenzoic acid, *p*-coumaric acid, and umbelliferone may all be considered as derivatives of phenol; protocatechuic acid, caffeic acid, aesculetin, and daphnetin are derivatives of catechol; vanillic acid, ferulic

COMPOUNDS IDENTIFIED IN WHEAT STEM RUST UREDOSPORES

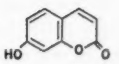
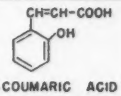
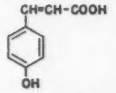
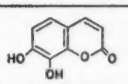
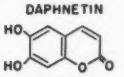
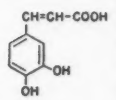
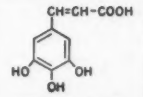
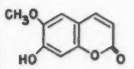
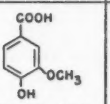
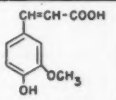
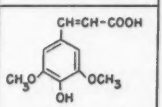
	COUMARINS	BENZOIC ACIDS	CINNAMIC ACIDS
NON-PHENOLIC	 COUMARIN		
MONOPHENOLS	 UMBELLIFERONE	 p-HYDROXYBENZOIC ACID	 o-COUMARIC ACID  p-COUMARIC ACID
o-DIPHENOLS	 DAPHNETIN  AESCULETIN	 PROTOCATECHUIC ACID	 CAFFEIC ACID
o-TRIPHENOLS		 GALLIC ACID	 SYRINGIC ACID
o-METHOXYPHENOLS	 SCOPOLETIN	 VANILLIC ACID	 FERULIC ACID
o-DIMETHOXYPHENOLS			 SINAPIC ACID

FIG. 1. Compounds identified in wheat stem rust uredospores.

acid, and scopoletin are derivatives of 2-methoxy phenol. Of these, only the 2-methoxy phenol derivatives appeared to be present to an appreciable extent as glycosides.

Effect on Uredospore Germination

These studies on germination were undertaken primarily to determine the relative inhibitory effects of the various compounds on spore germination, and if possible, to uncover something of the role they play in the physiology of wheat stem rust. Although no indoleacetic acid could be detected in rust spores it was considered advisable to check its action on germination also. All of the compounds listed in Table III were not available in sufficient quantity to include each in these tests. Most of those missing were the bound glycosides.

The preliminary survey indicated not only inhibition but in many instances marked stimulation of germination (Table IV). The compounds tested could be divided into two groups on the basis of their stimulatory or inhibitory action. In the first group increasing the concentration caused increased germination up to an optimum, which then declined at higher concentrations. Indoleacetic acid, coumarin, *o*-coumaric acid, protocatechuic acid, and umbelliferone belong in this group. In the second class are a group of acids which produced little or no stimulatory effect and several of them were markedly inhibitory even at 10 μg./ml. These acids are caffeic, *p*-hydroxybenzoic, ferulic, and vanillic. Caffeic acid has no effect below 200 μg./ml. and is not extremely toxic even at 1000 μg./ml. In the first test it appeared that there was a slight stimulatory effect from vanillic acid at 100 μg./ml. concentration but subsequent studies have not verified this observation.

The very marked stimulation to germination of spores caused by indoleacetic acid and coumarin at relatively high concentration levels raised several interesting questions and it was considered highly desirable to check at least a few compounds more thoroughly. Since macroscopic observation of the spore

TABLE IV

NUMBER OF SPORES GERMINATED AFTER 3 HOURS ON TEST SOLUTIONS

	Concentration, μg./ml.						
	1000	400	200	100	20	10	0
Indoleacetic acid	—	0†	83	232	37	30	30
Coumarin	0	0	52	*	*	*	30
o-Coumaric acid	0	5	44	90	10	16	30
Protocatechuic acid	0	0	14	160	95	75	30
Umbelliferone	0	0	0	5	83	73	30
Caffeic acid	10	15	37	30	55	27	30
Vanillic acid	0	0	20	62	25	22	30
p-Hydroxybenzoic acid	0	0	1	5	8	11	30
Ferulic acid	0	0	1	6	9	15	30

*Germination too high to permit accurate counting.
†A concentration of 500 μg./ml. was used.

film at 24 hours showed striking differences between treatments, another complete set of slides was prepared and photographed after 24 hours. The white spore mats indicate a high level of stimulation to germination (Fig. 2). It had become evident that, depending on age of uredospores from date of collection, the controls would vary greatly in percentage of spores which germinated. Lactophenol mounts of part of the spore film were therefore made from this second set of slides and counts were made on three to four hundred spores to determine the percentage germination. These results are presented in Table V. The same series of concentrations of all compounds was used here as in the first experiments except for *p*-hydroxybenzoic acid and ferulic acid. Since no germination had occurred at 1000 and 400 μg./ml., 5 and 1 μg./ml. levels were substituted. There was no stimulation to germination, however, and the results were very close to the control. In this series (Table V) ferulic acid-β-glucoside was tested without any marked effects at most levels of concentration. Results with most of the compounds confirmed the earlier tests (Table IV). Usually the percentage germination is higher at 24 hours than at 2 hours. In general on the water control with freshly collected spores (not over 1 week old) approximately twenty per cent of the spores had germinated after 24 hours' incubation. This was just sufficient to show as a faint white fuzz on the spore film. As germination increased to 50% or over, the mats became very white, and the brownish-red color of the original spore film disappeared entirely. In Table V germination of the controls varies from 7.5 to 23.9%. The compounds were tested in groups of two over a period of 2 months and it was not always possible to have

TABLE V

PERCENTAGE GERMINATION OF UREDOSPORES AFTER 24 HOURS ON TEST SOLUTION

	Concentration, μg./ml.						
	1000	400	200	100	20	10	0
Indoleacetic acid*	20.5	17.6	57.2	22.2	8.9	10.9	13.6
Indoleacetic acid	0.0	16.6	50.3	32.6	27.3	29.9	23.2
Coumarin	0.6	0.6	26.6	75.3	83.7	65.3	21.7
o-Coumaric acid	0.0	13.3	35.5	40.2	20.2	14.9	16.4
Protocatechuic acid	4.5	10.9	21.1	34.5	64.7	20.2	22.5
Umbelliferone	0.0	2.1	3.2	14.2	31.7	43.6	7.5
Daphnetin	6.7	11.8	13.2	32.2	44.1	50.8	7.5
Ferulic acid β-glucoside	0.0	6.7	3.8	12.7	8.1	19.6	15.6
Caffeic acid	2.6	8.7	11.5	9.6	8.0	7.0	17.9
Vanillic acid	0.0	5.3	24.2	23.2	21.1	22.9	18.1

	Concentration, μg./ml.						
	200	100	20	10	5	1	0
p-Hydroxybenzoic acid	22.3	23.0	20.8	20.9	22.5	24.7	23.9
Ferulic acid	0.4	1.7	11.1	11.3	9.4	18.6	21.1

*2 mg. spore load.

freshly collected spores available. The effect of age is clearly seen in the controls for umbelliferone and daphnetin where the spores were over 2 weeks old. These two compounds were tested the same day and the germination in two separate control slides was identical, but markedly lower than for younger spores.

Not only age of spores but the concentration of spores put on the surface of the 0.3 ml. water has a marked effect. In Table V two experiments with indoleacetic acid are shown, one with 2 mg. of spores, the other with the usual 1 mg. The double quantity of spores brings about marked inhibition of germination in the control as it drops nearly 50%. While the point of greatest stimulation still lies at 200 μg./ml., above this, inhibition is much less when the quantity of spores is doubled. Even at 1000 μg./ml. there was appreciable germination where 2 mg. of spores was used.

A few tests were also made on the effect of reducing the amount of spores from 1 mg. down to 0.5 mg. and finally 0.1 mg. on 0.3 ml. water, and water containing 200 μg./ml. of indoleacetic acid. These are shown in Table VI.

TABLE VI

EFFECT OF INDOLEACETIC ACID (200 μG./ML.) ON GERMINATION OF VARIOUS QUANTITIES OF 3-WEEK-OLD SPORES, READINGS STARTED AT 2.5 HOURS

Spore conc., mg.	Treatment	Ungerminated spores	Germinated spores	Per cent germinated
1.0	IAA	336.8± 6.3*	66.8±6.2	16.0
1.0	H_2O	380.8± 9.4	1.6±0.2	0.4
0.5	IAA	235.0±13.6	44.8±8.5	16.0
0.5	H_2O	242.0±10.5	1.6±0.4	0.7
0.1	IAA	233.4± 6.1	10.4±1.1	4.0
0.1	H_2O	183.8± 6.8	22.6±5.0	11.0

*Mean and significant difference for five samplings.

Readings were taken between 2 and 3 hours after the start of germination. Five different fields were selected near the center of the spore film and counts of all spores, germinated and ungerminated, with images lying within squares of the ocular micrometer were taken. Means and standard deviation were calculated and are presented in this table. While there was no difference in the response to 200 μg./ml. indoleacetic acid with the 1 mg. and 0.5 mg. spore load it was definitely inhibitory with 0.1 mg. of spores. The light spore load gave a higher percentage germination than either of the other concentrations of spores. The low percentage germination recorded for the controls in this experiment is attributed to the greater age of the spores used (nearly 3 weeks), to the early time of counting, and to the selection of fields in the center of the spore mass where germination is frequently lower than at the edge (see Fig. 2).

Discussion

The inhibitory action of coumarins and phenolic acids on the germination of the seeds of some higher plants has been described by Mayer and Evenari (24, 25), Sigmund (34, 35), and Kuhn *et al.* (22), and since these substances are

of widespread occurrence in plants, it has been concluded that they may play a role as germination and growth regulators (23). Nutile (27) has demonstrated that dormancy indistinguishable from that occurring naturally can be induced in non-dormant lettuce seeds by the use of coumarin, and there are increasing numbers of reports in the literature of the presence in seeds of inhibitory substances whose properties suggest that they may be members of this group. The factors recently reported by Wareing (38) and Black (5) to be responsible for the dormancy of *Xanthium* and birch seeds respectively, and Hemberg's inhibitor responsible for dormancy in the potato tuber (18) are in this category. A possible reason for the influence of these compounds on germination is the recently demonstrated role of mono- and di-hydric phenols in the formation and degradation of indoleacetic acid (28, 39). In the higher plants, this effect may lie in their ability to control the level of auxin in the seed. A relationship between inhibitor and auxin concentration in the peel of dormant potatoes has been proved (6, 37).

In the germination tests reported in this paper, an amount of spores was chosen to give a single closely packed layer on the surface of the water, and the volume of liquid was kept to a minimum. The number of spores per unit volume corresponded to the concentrations designated by Allen (2) as en masse. The marked depression of germination which he observed and showed to be due to the self-inhibitor present in the spores was found to occur. It was confirmed that an increase in germination counts could be obtained by diminishing the number of spores in the vessel. It may be assumed, then, that a certain concentration of the self-inhibitor had already been obtained in the solution and it might be expected that any addition of the same compound would further depress germination. It should therefore be possible to establish whether any of the substances found in rust spores are identical with the inhibiting compound. Of the compounds tested, only ferulic acid caused inhibition of germination at all concentrations down to 1 μg./ml. However, evidence presented by both Allen (2) and Forsythe (14) would seem to indicate that a part, if not the whole, of the germination of self-inhibiting factor present in uredospores is volatile. In one case (2) it could be removed from solutions by aeration while in the other (14) its effect was neutralized by the presence in the same vessel of a separate solution of silver nitrate. Of the substances found in the rust spores, only coumarin is sufficiently volatile to be active across an air gap, but its effect in stimulating germination of self-inhibited spores indicates that it is not the compound described. It seems more probable that an additional inhibitory substance, active at considerably lower dilution, may be formed by spores in contact with water through an aerobic process and that this has remained undetected in the work described here. Additional experiments are contemplated to resolve this question and to determine whether any of the compounds already identified have an inhibiting action when in contact with germinating spores which have either been depleted of self-inhibitor or are in concentrations low enough to be unaffected by the amount present.

The discovery that certain of the substances were capable of overcoming the effect of the inhibitor was unexpected. The mechanism through which they are able to effect a stimulation of germination is not known, but they are sufficiently dissimilar in chemical structure to make a purely chemical interaction unlikely. Since reversal of the inhibition at a biochemical level is more probable, the parallel effect obtained with indoleacetic acid is significant. It is tempting to speculate that this auxin may play a role in the physiology of uredospore germination. The mediating effect of phenolic compounds in the level of indoleacetic acid formation and degradation would also afford an explanation of the action of the other stimulatory substances. The view that the influence of indoleacetic acid on germination is due to the reversal of the depression caused by the self-inhibitor gained support from the fact that the level of auxin required for maximum stimulation varied according to the concentration of spores.

Peturson (29), examining the effect of different concentrations of indoleacetic acid on the germination of uredospores of the crown rust of oats, found little effect below 200 μg./ml. but inhibition at higher levels. The spore load was not specified, but was presumably small since germination of the controls was very high. Paper chromatography of an extract from 5 g. of uredospores failed to show any trace of indoleacetic acid using *p*-dimethylaminobenzaldehyde as a spray reagent. Shaw and Hawkins (30) used a more sensitive bioassay technique and were also unable to demonstrate the presence of indoleacetic acid in stem rust spores. Although recent evidence (17) suggests that indoleacetic acid does, in fact, take part in the normal metabolism of fungi, some doubt must therefore be expressed at present with regard to its role in uredospore germination. If its participation as an auxin should be proved it might throw some light upon the failure of uredospores to develop proliferating septate mycelia when germinated in artificial media where the regulating mechanism present in a susceptible host is absent.

The germination stimulator present in rust uredospores, described by Allen (1) and French *et al.* (15) as an unsaturated, neutral, somewhat volatile substance, soluble in water and organic solvents may, in fact, be coumarin. Its biological action, including the inhibition noted at higher levels, is also in accordance with the properties of this compound.

Coumarin and the phenolic compounds formed by wheat stem rust may play an important part in the physiological effects of rust attack on the host through their effect on indoleacetic acid metabolism. The production by the parasite of compounds which may stimulate the formation and/or retard the breakdown of indoleacetic acid, thus leading to a build-up of auxin level at the site of penetration, might explain the increased metabolism and migration of nutrients observed by Shaw and Samborski (31) at infection loci in susceptible hosts. Resistance might be conceived of as due to the ability of the host to neutralize the phenolic substance produced by the rust either by specific oxidases or glycoside formation. Alternatively, growth of the rust in a host possessing a high endogenous concentration of these compounds might rapidly

establish toxic levels at the site of infection, leading eventually to death of both the host cells and parasite. The number of phenols found in uredospores of race 15B, the differences in concentration, and their presence in both bound and unbound states suggest that the variety in types of resistance and infection observed might all find explanation. It may be noted that a correlation between phenolic content and rust resistance has already been demonstrated (11, 20, 26) and the role of protocatechuic, caffeic, and chlorogenic acids in the resistance of onions and potatoes to different infections has been described (3, 19, 21).

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THE USE OF BETA-PROPIOLACTONE FOR THE PREPARATION OF VIRUS VACCINES

I. SELECTION OF REACTION CONDITIONS[1]

JOHN R. POLLEY AND MURIEL M. GUERIN

Abstract

The use of beta-propiolactone (BPL) for the destruction of infectivity of influenza, mumps, and poliomyelitis virus suspensions has been investigated. It was found that: (1) the infectivity was destroyed more rapidly as the pH was increased from 6 to 8, and (2) the hemagglutination titers of influenza and mumps virus suspensions were most stable to BPL treatment at pH 7. Since the pH of the medium decreases with the hydrolysis of BPL, it was found desirable to use a buffered medium for BPL treatment. Influenza and mumps vaccines prepared with BPL have been lyophilized successfully for stable storage by the addition of 5% arginine or 5% peptone prior to lyophilization. Vaccines prepared from chick allantoic fluid, which had not been frozen, required dialysis of the virus concentrate prior to BPL treatment. Reaction conditions have been selected which render the virus suspensions noninfective while preserving most of the *in vitro* serological activity. Antigenicity tests of the virus suspensions treated with BPL are discussed in the second section (II) of this paper.

Introduction

One difficulty in the production of viral vaccines has been the preservation of antigenicity in the process of inactivation by formaldehyde. To overcome this difficulty, we have investigated a number of other virucidal agents in this Laboratory as possible replacements for formaldehyde. Many compounds have been screened for virucidal activity by Hartman *et al.* (1), and beta-propiolactone (BPL) was selected as the most promising agent for use on plasma from the standpoint of virucidal activity and low toxicity. Later, BPL was used for the preparation of some virus vaccines, prepared as 10% mouse brain suspensions (2) or in allantoic fluid (3).

To determine the possible usefulness of BPL in the preparation of vaccines in media other than 10% mouse brain suspensions, we have made a quantitative investigation of the effect of this reagent on the antigenicity and infectivity of influenza and mumps viruses. The optimal conditions for treatment found from this work are being applied to the preparation of experimental influenza and poliomyelitis vaccines, as described in the second part of this publication.

Materials and Methods

To determine the optimal conditions for treatment of influenza and mumps viruses by BPL, we investigated the effect of this reagent on the hemagglutination titer of these viral antigens. The influence of various suspending media on the stability of the hemagglutinin of vaccines under treatment with BPL was studied first. Also investigated were the effects of pH, reagent concentration, temperature, and duration of treatment.

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Contribution from the Virus Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

Effect of Suspending Medium

Freshly harvested influenza A (PR8) virus-infected allantoic fluid was centrifuged at 19,000 g. for 1 hour. The supernatant fluid was decanted and the pellets were resuspended in one-third of the original volume with physiological saline, isotonic phosphate buffer at pH 7, 2% albumen, human serum, or synthetic tissue culture medium (M150) (4). The BPL was prepared first as a 10% (v/v) aqueous solution at 4° C. This solution was added to the viral antigens to give a concentration of 0.5% in each. Samples of these treated antigens and untreated controls were placed in stoppered test tubes in water baths at 37° C. Portions were removed after various time intervals and tested for hemagglutination titer. The results are shown in Fig. 1. It can be seen that the stability of the hemagglutinin of the vaccine treated with BPL was influenced greatly by the choice of the suspending medium.

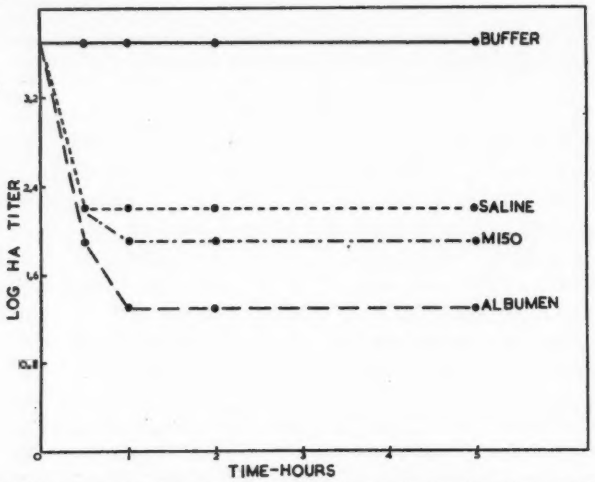

FIG. 1. Effect of suspending medium on the hemagglutination titer of influenza A virus treated with 0.5% BPL at 37° C.

Effect of pH

To determine the effect of pH on the stability of the hemagglutination titer of vaccines under treatment with BPL, we resuspended influenza A (PR8) virus in 0.25 *M* phosphate buffers at pH 6, 7, and 8. A concentration of 0.25% BPL was used and the vaccines were heated in a water bath at 37° C. Samples were removed after various time intervals and tested for hemagglutination titer. It was found that the titer was unchanged after 24 hours in the samples having a pH of 6 and 7 but it was reduced fourfold in samples with a pH of 8. Similar results were obtained when a concentration of 0.5% of BPL was used. When mumps virus was used, the hemagglutinin was somewhat more stable to treatment with BPL between pH 6 and 8 than was that of influenza A (PR8) but it decreased faster below pH 6. Since

synthetic tissue culture medium is the medium in which poliomyelitis vaccines are prepared, the previous experiment was repeated with this medium buffered to pH 7 with additional sodium phosphates to a concentration of 0.25 *M*. The titer remained stable during the 24 hours of incubation, indicating that this medium can be used successfully if buffered sufficiently.

Effect of BPL Concentration

The effect of the reagent concentration was studied by resuspending PR8 virus in isotonic phosphate buffer at pH 7. To samples of this virus suspension was added 10% aqueous BPL to give initial concentrations of 0.5, 0.25, and 0.1%. The samples were placed in rubber-stoppered test tubes and incubated in a water bath at 37° C. They were tested after various time intervals for hemagglutination titer. There was a marked loss in titer of the sample treated with 0.5% BPL but the samples containing 0.1 and 0.25% BPL showed no significant change in titer, which indicates that this medium is suitable for reagent concentrations up to 0.25%. It was shown in later experiments that with both influenza and mumps vaccines, the titer was unchanged after 7 days' storage at 37° C. with concentrations up to 1.0% BPL being used if the virus was resuspended in 0.25 *M* sodium phosphate buffer at pH 7 instead of in isotonic phosphate buffer of the same pH.

Destruction of Infectivity of Influenza and Mumps Viruses

While it has been shown in the experiments above that a pH of 7 is most suitable for maintaining the hemagglutination titer of vaccines treated with BPL, it remained to determine (1) the influence of the pH of the medium on the process of inactivation of the vaccine with BPL and (2) the optimal conditions of temperature, reagent concentration, and duration of treatment for the production of noninfective vaccines with maximum antigenicity. For this purpose, PR8 virus obtained from allantoic fluid stored in the frozen state was resuspended in 0.25 *M* phosphate buffers at pH 6, 7, and 8. The BPL was added as before to give a concentration of 0.1% in each sample. The tubes were placed in a water bath at 37° C. and tested after various time intervals for hemagglutination and infectivity titers. The infectivity tests were conducted by inoculating samples of the serially diluted vaccines into the allantoic cavity of 10-day-old chick embryos. The eggs were incubated for a further 72 hours. The allantoic fluids were tested for the presence of hemagglutinins. The results are shown in Fig. 2.

Similar experiments were conducted with PR8 virus suspended in synthetic medium M150 to which sufficient monobasic and dibasic sodium phosphates were added to give a concentration of approximately 0.25 *M* and a pH of 7 (0.28 g. Na_2HPO_4 + 0.07 g. NaH_2PO_4 per 10 cc. of medium). With this suspending medium it was found that the inactivation of the virus was more difficult to achieve. A concentration of 0.5% BPL rendered the virus noninfective after 2 hours; 0.25% BPL required 8 hours at least for inactivation; and 0.1% BPL was seldom satisfactory. The hemagglutination titers of the samples remained unchanged by the treatment.

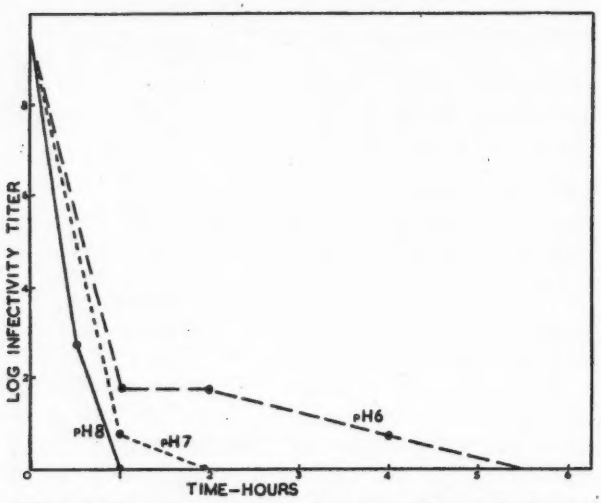

FIG. 2. Effect of pH on the rate of inactivation of influenza A virus treated with 0.1% BPL at 37° C.

The above experiments were repeated using mumps virus and similar results were obtained. Treatment at 4° C. was also investigated and was found to be unsatisfactory, as samples remained infective for at least 2 weeks, even when using a concentration of 0.5% BPL.

When these experiments were repeated with virus suspensions prepared by centrifuging freshly harvested allantoic fluid instead of allantoic fluid stored in the frozen state, it was found that the virus was more difficult to inactivate. An experiment was performed in which the pellets, obtained by centrifuging a pool of freshly harvested PR8 allantoic fluid, were resuspended (1) directly in 0.25 *M* sodium phosphate buffer at pH 7 or M150 or (2) in a small volume of saline, phosphate, or M150, dialyzed for 24 hours at room temperature against their respective media and then diluted to the desired volume or titer. Concentrations of 0.1 and 0.5% BPL were used and the tubes were incubated at 37° C. Samples were tested for infectivity after 24 hours. The importance of dialyzing virus concentrates prepared from freshly harvested allantoic fluid is shown in Table I.

Lyophilization of Influenza and Mumps Vaccines

For the purpose of storing prepared vaccine in a stable and convenient form, attempts were made to lyophilize it. Preliminary experiments indicated that if the treated vaccine was lyophilized directly, a serious loss of hemagglutination titer occurred. To determine whether vaccines treated with BPL could be lyophilized successfully by the addition of protective agents, PR8 virus was resuspended in 0.25 *M* sodium phosphate at pH 7 and treated with 0.5% BPL at 37° C. for 24 hours. Various reagents were then added to aliquots of the vaccine and samples of each were lyophilized in 3-cc. glass

ampoules, 1 cc. per ampoule, using an Edwards centrifugal freeze-drier. The samples were reconstituted to their original volume with distilled water and tested for hemagglutination titer. The results are shown in Table II. Samples of these vaccines were stored at 4° and 37° C. and tested after various time intervals to determine which reagents gave the most stable vaccines. After 3 months at 4° C., none of the samples showed any significant change in titer. At 37° C., however, only the samples containing 5% arginine or 5% peptone had retained their titer. The storage experiment is continuing.

TABLE I

EFFECT OF BPL ON INFECTIVITY OF PR8 VIRUS BEFORE AND AFTER DIALYSIS (RESULTS OF THREE SEPARATE EXPERIMENTS)

Virus suspended in:	Dialyzed against:	Dialyzed conc. diluted with:	BPL, %	Infectivity
PB*	—	—	0.1	+
PB	—	—	0.5	+
PB	PB	PB	0.1	—
PB	PB	PB	0.5	—
Saline	Saline	PB	0.1	—
Saline	Saline	PB	0.5	—
M150	—	—	0.5	+
M150	Saline	M150	0.5	—
M150	M150	M150	0.5	—

* 0.25 *M* phosphate buffer, pH 7.

TABLE II

EFFECT OF VARIOUS REAGENTS ON THE PRESERVATION OF THE HEMAGGLUTININ OF INFLUENZA A (PR8) VACCINE FOR LYOPHILIZATION

Compound	Conc., %	Titer
—	0 (Control, before freeze-drying)	5120
—	0 (Control, after freeze-drying)	160
Arginine	5	1280
Arginine	1	640
Glucose	5	2560
Albumen	1	10,240
Albumen	0.2	1280
Sucrose	5	2560
Sucrose	2.5	2560
Glycine	5	640
Polyvinyl alcohol	1	1280
Gelatin	1	2560
Gelatin	0.5	2560
Inulin	1	1280
Ascorbic acid	5	1280
Ascorbic acid	1	640
Cysteine HCl	5	2560
Cysteine HCl	1	2560
Peptone	5	10,240
Peptone	1	5120
Starch, soluble	1	640

Inactivation of Poliomyelitis Virus

To select a method of treatment for the inactivation of poliomyelitis virus, we tried a number of reaction conditions. The reaction conditions tried were those found successful for the inactivation of influenza and mumps viruses. For this purpose, type II (MEF1) poliomyelitis virus grown on monkey kidney cells cultivated in synthetic medium M150 was used. A common pool of this virus suspension was buffered to pH 7 with 0.25 *M* sodium phosphate as described above. This suspension was filtered through a Whatman No. 42 filter paper to remove coarse particles. Aliquots of it were treated with BPL under various conditions. After treatment, the samples were dialyzed for 24 hours at 4° C. against saline and then for 24 hours against M150. Dialysis was found to be necessary to decrease the concentration of the buffer which otherwise was toxic to the tissue culture cells. The entire sample was then tested for infectivity in tissue culture, two serial passages being used. The results are shown in Table III.

TABLE III

EFFECT OF TREATMENT WITH BPL UNDER VARIOUS CONDITIONS ON THE INACTIVATION OF TYPE II (MEF1) POLIOMYELITIS VIRUS

BPL conc., %	Temp., ° C.	pH	Time, days	Infectivity
0.25	37	8	1	−
0.5	37	8	1	−
0.25	37	7	1	±
0.5	37	7	1	−
0.25	22	8	7	−
0.5	22	8	7	−
0.25	22	7	7	+
0.5	22	7	7	−
0.5	4	8	7	−
0.5	4	7	7	±

NOTE: A minimum of three tests was done for each result above. The ± signifies noninfective on some tests but infective on others.

Results and Discussion

It is apparent from Fig. 1 that the stability of the hemagglutination titer of the vaccine is influenced by the suspending medium. On the other hand, the better stability of the hemagglutinin in a buffered solution suggested that the pH of the medium might be an even more important factor. This was confirmed when the effect of BPL treatment was studied in solutions buffered at various pH values. It was found that the hemagglutination titer of the virus suspension was most stable to BPL treatment at a pH of about 7. Thus, media such as M150 which were unsatisfactory as shown in Fig. 1 can be used successfully if buffered sufficiently. When the suspending medium is buffered, BPL can be used over a wide range of concentrations. With isotonic phosphate buffer at pH 7, BPL concentrations up to 0.25% can be used at 37° C.

without causing marked loss of hemagglutination titer. For employment of higher concentrations of BPL, increased buffer capacity is required; for example, concentrations of BPL up to 1.0% can be used when buffered in 0.25 *M* sodium phosphate at pH 7.

The investigation of the destruction of the infectivity of influenza virus by BPL gave several interesting results. Fig. 2 indicates that the infectivity is destroyed more rapidly as the pH of the vaccine is increased. Of practical interest also is the increasing tendency of the infectivity curves to "tail off" as the pH is decreased. An inactivation procedure which gives an infectivity destruction curve of the type found at pH 8 is obviously more certain to kill all the viable virus particles present than a procedure with an inactivation curve of the type shown for pH 6.

An important observation for vaccines prepared from chick allantoic fluid is the need for dialysis, as shown in Table I. It was found that the infectivity of virus suspensions prepared from freshly harvested allantoic fluid was more difficult to destroy than it was when the suspension had been prepared from allantoic fluid stored in the frozen state. When allantoic fluid is frozen and then thawed, a flocculent precipitate of urates, etc., is present. Centrifuging the supernatant fluid yields a virus pellet which is more pure than that obtained by centrifuging fresh allantoic fluid. Apparently, the impurities present in the virus pellets obtained on centrifuging freshly harvested allantoic fluid either protect the virus from the reagent or actually react with it and decrease its effect. This difficulty is overcome readily by dialyzing a concentrated suspension of the virus against saline for 24 hours. After dialysis, the virus concentrate is diluted to the desired volume or titer by the addition of the medium being used, and treatment is begun. Dialysis is not required for the treatment of poliomyelitis vaccines, since allantoic fluid is not involved.

As shown in Table II, it is necessary to add some protective agent to the vaccine before lyophilization to prevent a large loss of hemagglutination titer. It is evident that a considerable number of unrelated compounds will give the desired protective effect. However, when samples were stored at 37° C. and tested after various time intervals, it was apparent that a large variation existed in the ability of the different agents to confer stability on storage. Samples dried with sugars such as glucose and sucrose soon lost their titer. After storage for 3 months at 37° C., the only samples to retain their titer were those dried with 5% arginine or 5% peptone, as did a sample of the liquid vaccine stored in a sealed ampoule. However, when these dried samples were stored at 4° C. for the same length of time, no decrease of hemagglutination titer was observed.

As shown in Table III, of the 10 different conditions tried for the inactivation of poliomyelitis virus, seven produced noninfectivity as tested in tissue culture. As was the case with influenza A virus, a pH of 8 was superior to pH 7 for the destruction of infectivity. Similar results have been obtained when samples of a pool of types I, II, and III poliomyelitis viruses were treated under

these conditions. However, the selection of the best method of treatment for the preparation of a vaccine depends also on the antigenicity preserved in the different samples.

The antibody response obtained with influenza and poliomyelitis vaccines rendered noninfective by various procedures of treatment with BPL will be described in the second part of this report.

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THE USE OF BETA-PROPIOLACTONE FOR THE PREPARATION OF VIRUS VACCINES

II. ANTIGENICITY[1]

JOHN R. POLLEY AND MURIEL M. GUERIN

Abstract

An investigation has been made of the antigenicity of influenza virus suspensions rendered noninfective with beta-propiolactone (BPL). By treatment with BPL under various conditions, influenza vaccines could be prepared which were antigenic as indicated by production of specific antibody response in mice and guinea pigs. These vaccines were also immunizing as evidenced by their capacity to protect mice against challenge with live virus.

Introduction

In paper I of this series (2) it was shown that influenza and poliomyelitis virus suspensions can be rendered noninfective by the use of beta-propiolactone (BPL). With influenza virus suspensions the hemagglutination titer was most stable following treatment with BPL at pH 6–7 but the infectivity was destroyed more rapidly as the pH was increased to 8. From the data obtained from these experiments it was possible to select various procedures which would render the virus noninfective while still retaining most of the hemagglutination titer. That these inactivated virus suspensions would induce specific antibody responses remained to be shown. It is the purpose of this paper to present the results obtained when these experimental vaccines were inoculated into animals.

Materials and Methods

An influenza A (PR8) vaccine was prepared by resuspending the virus in 0.25 *M* phosphate buffer at pH 7, as described previously (2). This suspension was treated with 0.5% BPL at 37° C. for 24 hours. It was then lyophilized after the addition of 5% arginine. For use, the vaccine was reconstituted to its original volume with distilled water; it had a hemagglutination titer of 1:1280 and proved to be noninfective.

Serological Antibody Response

The first experiment was designed to determine (1) whether this lyophilized influenza vaccine prepared with BPL would induce a specific antibody response in guinea pigs and (2) which route of administration would result in the best response. Four groups of 12 guinea pigs each were inoculated by the intraperitoneal, subcutaneous, intramuscular, and intracutaneous routes, respectively. The dose in each case was 0.2 ml. Samples of blood were obtained at various time intervals after vaccination and tested by the hemagglutination–

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Contribution from the Virus Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa.

inhibition technique for specific antibody. The geometric mean of the individual results for each group was calculated, together with the standard error, and the results are shown in Table I.

TABLE I

INFLUENCE OF ROUTE OF INOCULATION OF INFLUENZA A (PR8) VACCINE PREPARED WITH BPL ON THE ANTIBODY RESPONSE*

Time after vaccination, weeks	Route of administration			
	Intraperitoneal	Subcutaneous	Intramuscular	Intracutaneous
4	3499 ± 346	538 ± 133	1280 ± 254	2153 ± 413
8	1280 ± 250	640 ± 0	1280 ± 0	1280 ± 0
12	380 ± 163	113 ± 73	226 ± 15	542 ± 16
20	211 ± 150	105 ± 48	113 ± 56	242 ± 210

*As measured by the hemagglutination–inhibition test.

Effect of Vaccine Titer on Level of Antibody Response

The next factor to be investigated was the relationship between the hemagglutination titer of the vaccine and the level of antibody induced. For this purpose, an influenza A (PR8) vaccine was prepared as before. The reconstituted vaccine had a hemagglutination titer of 1:2560 and was non-infective. Three further serial fourfold dilutions of this vaccine were prepared, giving four preparations with titers of 1:2560, 1:640, 1:160, and 1:40, respectively. These four vaccines were inoculated into four different groups of 10 guinea pigs each, the dose being 0.5 cc. by the intraperitoneal route, once a week for 3 weeks. Samples of blood were obtained at various time intervals after the last inoculation and the individual antibody levels were determined by the hemagglutination–inhibition test. The geometric mean of the individual titers of each group was calculated and the results are shown in Fig. 1.

Comparison of Vaccines Prepared by Different Methods

The antibody response to influenza vaccines, prepared with BPL by different methods, was next investigated. Freshly harvested allantoic fluid containing influenza A (PR8) virus was centrifuged at 19,000 g. for 1 hour. The precipitated virus was resuspended in about 1/40 of the original volume of saline and dialyzed at 4° C. for 48 hours against saline. One-half of this dialyzed viral concentrate was diluted with synthetic tissue culture medium (1), buffered at pH 8, until a final hemagglutination titer of 1:5120 was attained. The other half of the virus concentrate was diluted with tissue culture medium buffered at pH 6.5 until the same titer was achieved. Aliquots of these two virus suspensions were used for preparing the different BPL treated vaccines. After treatment with BPL, 5% arginine was added to each sample and the vaccines were lyophilized. For use, the dried vaccines were

reconstituted to their original volume with distilled water and tested for infectivity and hemagglutination titer. Only preparations proving to be noninfective to chick embryos were used for the animal tests. These vaccines were inoculated into guinea pigs, in a dose of 0.5 cc. by the intraperitoneal route. Each animal received a single inoculation only. Samples of blood were obtained at various time intervals after inoculation and tested for specific antibody by the hemagglutination–inhibition test. The results are shown in Table II.

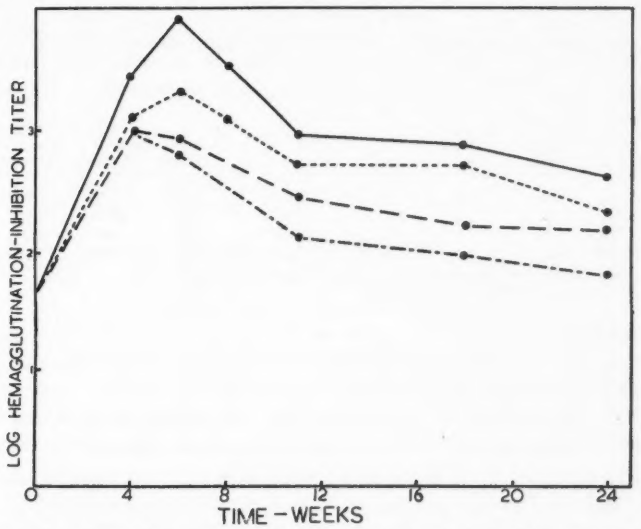

FIG. 1. Relationship between vaccine titer and the level of antibody produced. Top curve—vaccine titer 1: 2560; second from top—vaccine titer 1: 640; third from top vaccine titer 1: 160; bottom curve—vaccine titer 1: 40.

TABLE II

ANTIBODY RESPONSE IN GUINEA PIGS TO A SINGLE INOCULATION OF INFLUENZA A (PR8) VACCINES PREPARED WITH BPL UNDER VARIOUS EXPERIMENTAL CONDITIONS

Conditions of treatment				Resultant vaccine titer*	Antibody titer† after:			
BPL conc., %	Temp., °C.	Time, days	pH		4 weeks	10 weeks	18 weeks	24 weeks
0.5	45	¼	8	1280	6053‡	3041	1077	905
0.5	37	1	8	2560	5030	5090	1076	626
0.5	22	7	8	2560	7155	3620	1810	874
0.5	45	¼	6.5	640	1077	2560	380	85
0.5	37	1	6.5	2560	7112	3045	1077	785
0.5	22	7	6.5	2560	2152	3045	905	342
0.1	45	¼	8	1280	7112	5120	1810	848
0.1	37	1	8	1280	8460	4310	1210	910
0.1	45	¼	6.5	1280	8460	6053	1810	1232

*As measured by the hemagglutination test.
†As measured by the hemagglutination–inhibition test.
‡Geometric mean of six individual titers for each value shown.

In an experiment similar to that described above, four influenza A (Shope swine virus strain) lyophilized vaccines were prepared by four different procedures using BPL. The reconstituted vaccines were noninfective and were inoculated in 0.2 cc. amounts intraperitoneally into four different groups of 60 mice each. At various time intervals after inoculation, 10 mice were selected at random from each group and tested by the hemagglutination–inhibition test for specific antibody. The results are shown in Table III.

TABLE III

ANTIBODY RESPONSE IN MICE TO THE INTRAPERITONEAL INOCULATION OF 0.2 CC. OF INFLUENZA A (SHOPE VIRUS STRAIN) VACCINES PREPARED WITH BPL

Conditions of treatment				Resultant vaccine titer	Antibody titer* after:			
BPL conc., %	Temp., °C.	Time, days	pH		2 weeks	8 weeks	18 weeks	24 weeks
0.5	37	1	8	1280	160	640	640	160
0.5	37	1	7	2560	320	2560	1280	320
0.1	45	½	8	2560	320	1280	1280	320
0.1	37	1	8	2560	320	1280	640	320

*As obtained in the hemagglutination–inhibition test.

Challenge by Live Virus

The experiments described above demonstrated that influenza vaccines, prepared with BPL under various experimental conditions, induced specific antibody response in mice and guinea pigs. It remained to be demonstrated that such vaccines would confer protection against challenge by live virus.

For this purpose, an influenza A virus, Shope's swine influenza strain adapted to mice, was used for the protection tests. Intranasal inoculation of this virus into normal adult mice causes death in 4–10 days. This virus had been maintained by alternate egg and mouse passage. Egg passage was carried out by the inoculation of the virus at a dilution of 10^{-4} into the allantoic cavity of 10-day-old chick embryos. The eggs were incubated for 72 hours, after which the virus-infected allantoic fluid was harvested. Mouse passage was carried out by the intranasal inoculation of this allantoic fluid into adult mice. The mouse lungs were harvested 3–4 days later and prepared as a 20% saline suspension for repassage in eggs.

In the first challenge experiment, a swine influenza vaccine was prepared in a similar manner to that used for the PR8 vaccines described above. The method of treatment was 0.5% BPL at pH 7 for 24 hours at a temperature of 37° C. The vaccine was lyophilized with 5% arginine as before. The virus suspension had a titer of 1: 2560 before treatment; after reconstitution to original volume with distilled water the vaccine had a titer of 1: 1280 and was noninfective. The reconstituted vaccine was inoculated into three groups of 30 mice each, with each mouse receiving 0.5 cc. by the intraperitoneal route. Ten days later, the mice were reinoculated with a similar dose of vaccine. As controls, three groups of 30 mice each were inoculated similarly using normal allantoic fluid treated with BPL. One week after the final inoculation,

the mice were challenged with live virus by the intranasal route. Three serial fivefold dilutions of the challenge virus suspensions were prepared, each of which was inoculated into 30 vaccinated and 30 control mice. The results of this experiment are shown in Table IV.

TABLE IV

THE PROTECTIVE EFFECT IN MICE OF INFLUENZA A (SHOPE SWINE INFLUENZA STRAIN) VACCINE PREPARED WITH BPL

Dilution of challenge inoculum	Controls	Vaccinated
1:5	3/29*	30/30
1:25	6/30	29/29
1:125	10/30	29/29

* Numerator = number of survivors.
Denominator = number of mice on test.

In another experiment the effect of spacing of the inoculations was investigated. A swine influenza vaccine with hemagglutination titer of 1:640 was prepared as above. Four groups of 80 mice each were inoculated with 0.5 cc. of this vaccine by the intraperitoneal route at each inoculation, the schedule being as follows: Group I received a single inoculation, Group II received three inoculations at intervals of 1 week, Group III received three inoculations at intervals of 2 weeks, and Group IV were inoculated three times at intervals of 1 month. The inoculations were timed so that all mice received their last injection on the same day. One month later, 30 mice of each vaccinated group and 30 control mice were challenged with a 1:10 dilution of the inoculum used in the former experiment. In addition, 10 mice from each group were selected at random and the antibody levels determined. This challenge procedure was repeated on 30 mice of each group after 5 months. The results are shown in Table V.

TABLE V

SURVIVORS OF CHALLENGE EXPERIMENT IN MICE INOCULATED WITH INFLUENZA A (SHOPE SWINE INFLUENZA) VACCINE IN SINGLE AND VARIOUSLY SPACED TRIPLE DOSES

Group No.	Immunization schedule	Challenged after 1 month		Challenged after 6 months	
		Antibody titer	Survivors	Antibody titer	Survivors
I	1 inoc.	160	28/30	160	18/30
II	3 inoc. at weekly intervals	1280	28/30	640	25/30
III	3 inoc. at 2-weekly intervals	1280	28/30	1280	25/30
IV	3 inoc. at 4-weekly intervals	1280	28/30	1280	26/30
Control		20	8/30	20	6/30

Discussion

From Table I it can be seen that the influenza vaccine prepared with BPL has induced a significant specific antibody response in guinea pigs. After 1 month, the antibody response was considerably higher in the animals inoculated by the intraperitoneal and intracutaneous routes. As the time following vaccination increased, the antibody levels in the four groups decreased in a parallel manner, so that after 20 weeks the antibody levels induced by the intraperitoneal and intracutaneous routes were still higher than in the other two groups. The fall in antibody level in all groups was fairly rapid and suggests the need for booster inoculations, as proved in the later experiments.

From Fig. 1 it is apparent that the four vaccines have initiated similar rates of antibody response, the peak occurring 4–6 weeks after the last inoculation. The rate of decrease in antibody levels was similar with the four vaccines, as was noted in the experiment above.

In Table II are shown the results when influenza A (PR8) vaccines prepared with BPL under various conditions were compared on the basis of their ability to provoke a specific antibody response. It is evident that numerous methods can be used to produce virus suspensions which are noninfective and still retain most of their hemagglutination titer. That these virus suspensions are also antigenic is shown by the level and the duration of the antibody response following a single inoculation. It may also be noted that the antibody levels produced in this experiment, wherein 0.5 cc. of inoculum was administered, are significantly higher than those shown in Table I where a 0.2 cc. dose was used. In general it appears that influenza virus suspensions which retain a high level of hemagglutination titer after being rendered noninfective with BPL can be expected to induce an antibody response. Since the hemagglutination titer gives some indication of the immunizing capacity of a vaccine it is therefore a most useful procedure in screening different methods of vaccine preparation. On the other hand, different vaccines in this series, which possessed the same hemagglutination titer, provoked different levels of antibody response. This indicates that, within the limits of the experimental error of these methods, the hemagglutination titers of different vaccine preparations will not serve as a true measure of their antigenicity.

When different vaccines of swine influenza virus, prepared with BPL under various conditions, were inoculated into mice, they also induced specific antibody responses. In this experiment, the antibody levels which resulted are correlated more closely with the hemagglutination titers of the vaccines inoculated.

In Table IV, it is shown that a swine influenza virus vaccine prepared with BPL is also capable of conferring protection against challenge by the live mouse-adapted virus. The three dilutions of virus inoculum used to challenge the mice were relatively lethal for the unvaccinated mice, whereas the vaccinated groups showed the protective effect of the vaccine.

In another experiment, as shown in Table V, the protective effect of the vaccine against challenge by live virus is again clearly demonstrated. Even 6 months after the last inoculation there is good protection against live virus. The results 1 month after the course of vaccinations indicate no correlation between the antibody level as measured in the hemagglutination–inhibition test and the degree of protection afforded by the vaccine, unless an antibody level of 1:160 is indicative of good protection.

These experiments have shown that (1) beta-propiolactone can be used to render influenza virus suspensions noninfective while preserving most of the hemagglutination titer, (2) various reaction conditions can be selected to achieve this purpose, (3) in this investigation, maintenance of a high level of hemagglutination titer after treatment gave reason to expect antigenicity, (4) by treatment with BPL influenza vaccines could be prepared which were antigenic as indicated by production of specific antibody response in mice and guinea pigs, (5) these vaccines were also immunizing as evidenced by their capacity to protect mice against challenge with live virus.

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METABOLISM OF RHIZOBIA IN RELATION TO EFFECTIVENESS[1]

H. KATZNELSON AND A. C. ZAGALLO[2]

Abstract

The ability of effective and ineffective strains of rhizobia to degrade 6-phosphogluconate directly to pyruvate and the metabolic activity of these strains in relation to the glycolytic, and hexose monophosphate oxidative pathways of carbohydrate break down were studied. No distinct relationship between metabolic activity, pyruvate production, and effectiveness was obtained although it was found that effective strains of rhizobia oxidized succinate more rapidly than ineffective strains. The organisms possess certain enzymes involved in glycolysis as well as alternate pathways for hexose phosphate utilization via the oxidative route.

Introduction

Strains of rhizobia, the root nodule bacteria of leguminous plants, differ widely in their effectiveness as symbionts in the fixation of atmospheric nitrogen. For this reason many attempts have been made along metabolic, nutritional, and cultural lines to distinguish between effective and ineffective types (1, 3, 5, 6, 7, 10). Since it was reported recently (8) that cell-free preparations of two species of *Rhizobium* had in common with a number of phytopathogenic bacteria, the ability to degrade 6-phosphogluconate to pyruvate, it was considered of interest to compare the capacity of effective and ineffective strains to carry out this reaction. Concurrent studies were conducted on the metabolic activity of these strains on a number of common substrates, and on the presence in cell-free preparations of certain respiratory enzymes involved in the glycolytic, and hexosemonophosphate oxidative pathways of carbohydrate break down with a view to distinguishing between effective and ineffective cultures. The results of this work are reported in this paper.

Materials and Methods

Representative effective and ineffective strains were selected from the Bacteriology Division Culture Collection and were maintained on a 1% glucose – inorganic salts agar medium containing 0.15% yeast extract (Difco). Cells for metabolic work were grown in this medium (without agar and containing 1% yeast extract) in Fernbach flasks on a rotary shaker for 48 hours at 28° C.; they were then harvested and washed twice with tap water. For manometric studies the cells were suspended in $M/15$ phosphate buffer at pH 7.0 to give a turbidity equivalent to a reading of 400 in the Klett–Summerson photoelectric colorimeter with a 660 mμ filter. Two milliliters of cell suspension were used per Warburg vessel, which contained also 0.2 ml. 20% KOH in the center well and 0.1 ml. $M/20$ substrate in the side arm.

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Cell-free preparations were obtained by suspending cells in four times their wet weight of distilled water and treating them in a Raytheon 10 kc. oscillator for 5 to 10 minutes. The suspension was centrifuged for 10 minutes at 10,000 r.p.m. and the supernatant fluid used. Double arm Warburg vessels were employed to determine oxygen uptake by these sonates. Each vessel contained 1.5 ml. sonic extract and 0.5 ml. *M*/15 veronal buffer (pH 7.0); 0.2 ml. 20% KOH was placed in the center well. One side arm contained 0.1 ml. *M*/20 substrate, the other 0.3 ml. of a mixture of cofactors: 10 μM. adenosine–triphosphate (ATP) and 0.5 μM. each of di- and tri-phosphopyridine nucleotide (DPN and TPN). After equilibration, substrate was tipped into the main compartment and readings taken at 15-minute intervals for 75 minutes, after which the cofactors were added.

Metabolic gas exchange was determined at 27.5° C.; endogenous values were subtracted in all cases. Pyruvate production was measured by the direct or double extraction procedure of Friedmann and Haugen (4). Protein was estimated by means of the Folin–phenol reagent as described by Lowry *et al.* (9). Reduction of DPN or TPN was followed at 340 mμ in a Beckman DU spectrophotometer. Aldolase and phosphohexokinase activity was determined by the method of Sibley and Lehninger (11).

The following substrates used were obtained commercially: glucose (G), gluconate (GA), 2-ketogluconate (2KGA), glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-diphosphate (F-1,6-diP), acetate, pyruvate, succinate, aspartate, and glutamate. Six-phosphogluconate (6PG) and a partially purified preparation of 2-keto-3-deoxy-6-phosphogluconate (KDPG) were kindly supplied by Dr. W. A. Wood, University of Illinois, Urbana, and Dr. M. Doudoroff, University of California, Berkeley, respectively. The barium salts of the hexose phosphates were converted to ammonium salts whereas the remaining acids were used as the sodium salts. All solutions were adjusted to pH 7.0.

Experimental Results

The results obtained with intact cells are summarized in Table I. Succinate and glucose were utilized readily by most of the strains tested. Oxidation of the remaining substrates varied with the cultures used. No clear-cut correlation between respiratory activity and strain effectiveness was evident except with succinate, which was oxidized more rapidly in every case by the effective strains. A similar trend occurred with pyruvate although the Qo_2 values were much lower than those for succinate.

The experiments with cell-free extracts yielded little evidence of metabolic differences between effective and ineffective strains. Succinate again was utilized rapidly (in the absence of cofactors) little activity being observed with the other substrates until the cofactors were added (Fig. 1). At this time, however, oxygen uptake occurred suggesting the presence in the sonates of enzymes such as a TPN- or DPN-linked dehydrogenases, kinases, and other respiratory enzymes.

TABLE I

OXIDATION (Q_{O_2}) OF VARIOUS SUBSTRATES BY *Rhizobium* SPECIES*

Substrate	*R. meliloti*		*R. leguminosarum*		*R. phaseoli*	
	787 (−)†	832 (+)	780 (−)	312 (+)	775 (−)	777 (+)
Glucose	5	15	22	16	23	35
Gluconate	4	6	31	27	10	18
2-Ketogluconate	4	0	2	16	3	2
Glucose-6-PO_4	4	4	3	3	4	6
Acetate	10	12	21	15	10	9
Pyruvate	5	11	6	12	4	8
Succinate	29	50	42	64	12	45
Aspartate	6	21	34	3	16	5
Glutamate	17	37	32	3	25	4

*Q_{O_2} = μl. oxygen uptake per mg. dry cells per hour.
†(−) = Ineffective strains.
(+) = Effective strains.

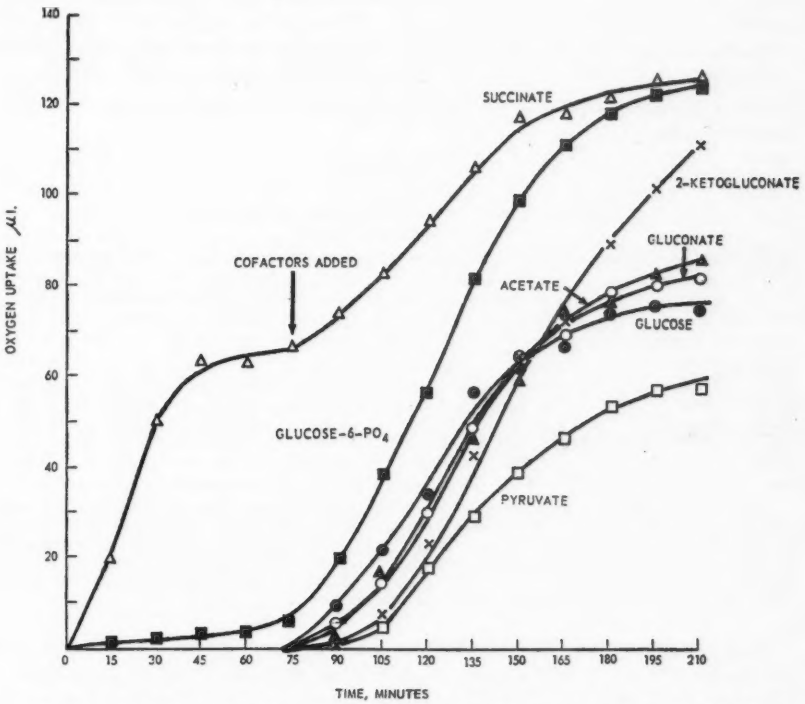

FIG. 1. Oxidation of various substrates by a cell-free preparation of *Rhizobium phaseoli* as affected by cofactors (ATP, TPN, and DPN).

The ability of sonates of effective and ineffective strains to produce pyruvate from 6PG is shown in Table II. This reaction was also used to determine the presence of other enzyme systems concerned in hexose metabolism by coupling the specific enzymatic reaction (isomerase, kinase, etc.) with the TPN-specific G6P dehydrogenase occurring in these sonates, to produce 6PG. Thus for example the presence of phosphohexose isomerase could be deduced if F6P yielded pyruvate following TPN addition, the sequence being F6P $\rightleftharpoons$ G6P $\rightleftharpoons$ 6PG $\rightarrow$ KDPG $\rightarrow$ pyruvate. Similarly the presence of hexokinase could be deduced if pyruvate were obtained from glucose + ATP + TPN or of gluconokinase if pyruvate were obtained from gluconate + ATP since 6PG would be formed in both instances. The results suggest that phosphohexose isomerase (F6P + TPN), G6P dehydrogenase (G6P + TPN), hexokinase (glucose + ATP + TPN), and gluconokinase (GA + ATP) were present in these sonates, the last named being the most active. There was no evidence for the presence of phosphoglucomutase (G1P + TPN) by this method. It is clear that enzyme activity varied considerably; however, no attempt was made to carry these reactions out under optimum conditions for each specific system. The amount of pyruvate produced from 6PG itself was considerably below that which is theoretically possible from 5 μM. of this compound. This may be explicable on the ground that no attempt was made to prevent the further utilization of the pyruvate thus produced. No relationship between strain effectiveness and enzyme activity was apparent from these results even after recalculation on a unit protein basis.

TABLE II

PYRUVATE PRODUCTION FROM VARIOUS SUBSTRATES BY *Rhizobium* SPECIES*

Substrates and cofactors	*R. meliloti*		*R. leguminosarum*		*R. phaseoli*	
	787 (−)	832 (+)	780 (−)	312 (+)	775 (−)	777 (+)
G1P + TPN	0	0	0	0	0	0
F6P + TPN	0.69	0.54	0.86	1.14	0.75	0.54
G6P + TPN	0.30	0.66	0.88	0.90	0.80	0.30
G + ATP + TPN	0.57	0.56	0.79	0.50	0.54	0.57
GA + ATP	2.80	2.10	2.30	1.55	2.30	1.75
6PG	2.80	2.25	2.7	2.52	3.25	2.45

* Results are expressed as μM. pyruvate. The reaction mixture contained: 0.3 ml. sonic extract 0.1 ml. *M*/1 tris (hydroxymethyl) aminomethane buffer pH 7.7; 0.1 ml. *M*/10 $MgCl_2$; 0.2 ml. ATP (100 μM. per ml.); 0.2 ml. TPN (1.4 μM. per ml.); 0.1 ml. *M*/20 substrate (5 μM.). It was incubated for 30 minutes at 35° C. and the reaction stopped by the addition of 1 ml. of an 0.1% solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. Appropriate blanks were used and their values subtracted.

Experiments were also conducted with extracts from an effective and an ineffective strain of rhizobia to determine if KDPG, the intermediate in the Entner–Doudoroff (2) 6PG-splitting system, could be metabolized. An appreciable amount of pyruvate (approximately 0.3 μM. per μM. KDPG

preparation) was obtained, thus supporting the hypothesis that these bacteria can use this pathway of hexose phosphate metabolism. Absolute pyruvate values could not be obtained with this partially purified substance.

Further confirmation of the presence in the sonates of an active TPN-linked G6P dehydrogenase may be noted in Table III. DPN was not reduced under these conditions but the presence of DPNH oxidase in these sonates was not excluded. A somewhat less active TPN-linked 6PG dehydrogenase and weak hexokinase activity were also detected by the spectrophotometric method. The low pyruvate values obtained in the presence of TPN (Table II) may be due therefore to the utilization of some 6PG via the pentose pathway.

The results of tests to demonstrate the presence of aldolase and phosphohexokinase in these sonates are shown in Table IV. A very active aldolase and a considerably less active kinase were found. Again the absence of any correlation between enzyme activity and strain effectiveness is noteworthy.

TABLE III

REDUCTION OF TRIPHOSPHOPYRIDINE NUCLEOTIDE (TPN) BY SONIC EXTRACTS OF RHIZOBIA*

Substrates and cofactors	Q_{OD}†					
	R. meliloti		*R. leguminosarum*		*R. phaseoli*	
	787 (−)	832 (+)	780 (−)	312 (+)	775 (−)	777 (+)
G6P + TPN	9.6	11.3	13.9	11.3	12.0	13.4
6PG + TPN	2.3	2.4	3.2	4.1	4.8	1.0
G + ATP + TPN	1.0	1.3	1.0	1.7	1.0	1.0

* The reaction mixture in the cuvette contained 0.01 to 0.1 ml. sonic extract depending on activity; 10 μM. substrate; 0.4 ml. glycylglycine buffer (*M*/4, pH 7.4); 0.3 ml. $MgCl_2$ (*M*/10); 0.2 ml. TPN (1.4 μM. per ml.); 0.2 ml. ATP (100 μM. per ml.); water to 3.0 ml.
†Q_{OD} = Change in optical density at 340 mμ per mg. protein per hr.

TABLE IV

COMPARISON OF ALDOLASE AND PHOSPHOHEXOKINASE ACTIVITY OF SONIC EXTRACTS OF RHIZOBIA*

Organism	Aldolase (diluted 1 : 2)	Phosphohexokinase (undiluted)
R. meliloti 787 (−)	0.70	0.32
" 832 (+)	0.54	0.17
R. phaseoli 775 (−)	0.62	0.18
" 777 (+)	0.66	0.18

* Expressed as optical density at 540 mμ. The reaction mixture was that of Sibley and Lehninger (11); F-1,6-diP was used as substrate for the adolase test whereas F6P, ATP, and $MgCl_2$ were used in the test for phosphohexokinase

Discussion

The purpose of this study was to determine if a relationship could be found between the metabolic activity of intact cells and cell-free extracts of rhizobia, and their effectiveness as symbionts in the process of nitrogen fixation. The only consistent relationship among the strains tested was the ability of intact cells of effective strains to oxidize succinate more rapidly than that of ineffective strains. However, these results can only be considered significant if duplicated with a larger number of strains. It is of interest that "good" strains of *Rhizobium leguminosarum* were found by Tam and Wilson (12) to dehydrogenate succinate, lactate, formate, glycerol, sorbitol, raffinose, maltose, and fructose more rapidly than "poor" strains of *R. trifolii*. However, these results may be due to species rather than to strain difference. The authors themselves concluded that the differences obtained were "not consistently associated with either effectiveness or ineffectiveness of organism". Jordan (6) obtained no evidence of any "fundamental differences in L- and D-amino acid transaminating ability that might be linked to the condition of parasitism or effectiveness."

The presence of a variety of enzymes of both the glycolytic and hexose-monophosphate pathways of hexose break down has been indicated although again no striking differences between effective and ineffective strains were encountered. That alternate systems for the metabolism of hexose phosphates are present in these organisms is evident. It has been shown for example that in the absence of TPN, 6PG produces pyruvate, presumably by the Entner–Doudoroff (2) route via the intermediate KDPG, which is also degraded to pyruvate. On the other hand, on the addition of TPN, reduction of 6PG occurs and preliminary tests have shown the presence of pentose in this system.

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FAVUS OF MICE[1]

F. BLANK

Abstract

Isolated cases and family epidemics of mouse favus were found in the lower St. Lawrence Valley, especially in the Kamouraska County. The specific agent of mouse favus was repeatedly isolated from animals (mice, cats, dogs, fox) and children and adults (smooth skin and scalp). The mouse is the normal host of the dermatophyte. Cats and dogs acquire the disease from mice and transmit it to man.

Cultures of the isolated dermatophytes resembled in all respects Quincke's α-Pilz (*Achorion quinckeanum*). The formation of elongate microconidia and pedicellate, spindle-shaped, thick-walled macroconidia with pointed tips was observed in slide cultures made with wort agar (Difco). These morphological features place the fungus in the genus *Microsporum*. Therefore, the specific agent of favus of mice should be named forthwith. *Microsporum quinckeanum*.

I. Introduction

Draper, according to Sabouraud (73), was the first who observed favus in mice in 1854. Gluge and d'Udekem (35) and Friedreich (32) reported favic lesions in mice in 1857 and 1858, respectively. The latter author made the observation, remarkable at that time, that the fungal elements of the scutula of mice were smaller than those of human favus. Two other reports on favus of mice followed shortly afterwards. Zander (96) observed scutula on the muzzle and the ears of a mouse and on the abdomen and extremities of a cat. This author confirmed the earlier observations of Friedreich (32) on the width of the hyphae of the causative organism. In the same year, Schrader (78) described the presence of numerous favic mice in a house in Hamburg. Simon (81, 82) also made a report from Hamburg on mice affected with favus.

Quincke (67) is usually credited with having isolated the fungus causing favus of mice first, in 1886, although, according to Zopf (97), Nicolaier might have had cultures before Quincke. However, the only record of the results of Nicolaier's investigations were in C. Flügge, Die Mikroorganismen, Leipzig, 1886. Quincke (67) named this dermatophyte α-Pilz. Two other fungi isolated from favic lesions were called β- and γ-Pilz. Quincke's γ-Pilz represented the fungus isolated from human favus, while the β-Pilz was later regarded as not being a valid species. The α-Pilz had been isolated from the neck of a man who used to carry flour bags. In a later paper, Quincke (68) reported human infections probably contracted from mice. He believed also that cats might become infected by contact with mice and then transmit the disease to man. Quincke gave a perfect description of the dermatophyte and the lesions caused by it. He compared the agents of favus of mice (α-Pilz) and of human favus (γ-Pilz, *Trichophyton*

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Contribution from the Department of Bacteriology and Immunology, McGill University, Montreal, Quebec.

Schönleini) and emphasized the distinctive features of these two fungi. Boer (13) isolated a dermatophyte from mice. The cultures he obtained resembled those isolated from human favus whereas the description of the microscopic features was similar to that of Quincke's α-Pilz.

Zopf (97) named Quincke's α-Pilz *Oidium quinckeanum* and not *Achorion quinckeanum* as stated by Bodin (11) and Vanbreuseghem (92). Sabouraud (73) attributed *Achorion quinckeanum* to Bodin. Blanchard (8) apparently was the first to place this fungus in the now defunct genus *Achorion*.

Frank (31) isolated a dermatophyte from a favic mouse. The fungus corresponded in almost every respect to the α-Pilz; however, Frank did not find the macroconidia observed by Quincke. The author again emphasized the differences between the fungi isolated from human and mouse favus and concluded that forthwith favus should be regarded as a clinical entity only. This was a very unpopular statement at that time. Unna (91) and Neebe and Unna (56) also believed in the plurality of the causative organisms of favus. Busquet (15) assumed that there was only one dermatophyte causing favus and that the α-Pilz of Quincke represented only a changed growth form that was due to a prolonged parasitic stay in mice. Bodin (11) made a new study of the dermatophyte after he had isolated it from mice and man. He recognized his cultures as identical with the α-Pilz and confirmed Quincke's mycological findings. Although favus of mice may be caused by several different dermatophytes, Bodin expressed the wish that the denomination of the specific agent of mouse favus should be reserved for *Achorion quinckeanum*. Wandel (94) investigated six mice with favic lesions in 1903. One mouse was found in the same building in Kiel in which Quincke had found a cat infected by *A. quinckeanum* many years before. *A. quinckeanum* was isolated from animals and some human cases as well. The description of the cultures, especially that of the macroconidia, corresponds well with our own findings, which we shall describe later.

Bloch (9), in 1908, studied the immunological behavior of *A. quinckeanum* and came to the conclusion that this dermatophyte was more closely related to the *Trichophyton* species *sensu* Sabouraud than to the agent of human favus called at that time *A. Schönleini*. In this respect, he shared the opinion of Bodin (11), who had expressed similar views based on his mycological studies.

Plaut (64), too, made a clear distinction between the fungi causing favus in mice and those causing this syndrome in man. He called Quincke's α-Pilz and γ-Pilz "Flaumtyp" and "Wachstyp", respectively. Tomasczewski (88) studied strains isolated by Bloch (10), Bodin (11), and Chajes (17). He confirmed Quincke's observations and again stressed the differences between *A. quinckeanum* and *A. Schönleini* so often refuted by other authors. One reason for the reluctance to accept Quincke's and Bodin's findings was that some authors (24, 25, 27, 45, 62, 63) isolated nothing but *A. Schönleini* from favic lesions simply because infections due to *A. quinckeanum* were extremely rare as compared with the high incidence of *A. Schönleini* infections in Europe at that time. Another reason for their reluctance was the peculiar

clinical behavior of *A. quinckeanum* (superficial lesions of the smooth skin, scutulum formation restricted to the smooth skin, no invasions of hairs) which did not fit into the doctrinaire schemes of the dermatologists.

In the more recent literature the agent of mouse favus has been either neglected, lumped with other dermatophytes, or even completely disregarded (18, 33, 49, 84). Therefore, we wish to report our mycological and epidemiological investigations on favus of mice in the lower St. Lawrence Valley. These confirm earlier reports, and also reveal new information on favus of mice, and its causative organism, especially as to its taxonomic position.

II. Mycological Investigations

About thirty mice (kindly identified as *Mus musculus domesticus* Rutty by J. D. Cleghorn, Curator of Zoology, Redpath Museum, McGill University) showing skin infections were caught by cats in a barn in Kamouraska, Que., between August 1955 and January 1956. Hay and oats were stored in the barn. All mice had scutula on the muzzle, ears, or back (Figs. 1 and 2). Some mice had lesions on the extremities or tail from which the fungus growth could easily be scraped off with a scalpel.

Autopsies of four affected mice were done by Dr. G. C. McMillan, Department of Pathology, McGill University, to whom we wish to express our gratitude. The presence of any fungal elements could not be demonstrated in any internal organ.

Scrapings taken from the scutula of the mice were inoculated on Sabouraud glucose agar, and the tubes were incubated at 25° C. Growth was visible after only 24 hours, whereas growth from subsequently cultured canine and human species became visible after 48 and 72 hours, respectively. The colonies developed rapidly. At first, they were flat and adhered to the medium in the same way as those of *Microsporum canis*. Then concentric rings appeared around a small raised center. Later, deep radiating furrows developed and the culture appeared raised and folded. The borders of the colonies were characterized by small projecting fringes of the aerial mycelium. The dense aerial mycelium was snow-white and the colonies resembled cultures of dermatophytes which had become pleomorphic. The reverse of the colonies was yellowish-brown (Fig. 3).

The fungus showed the same development and colonial appearance on Sabouraud maltose agar.

Cultures grown on Sabouraud glucose agar at 25° C. and then incubated at 37° C. developed a purplish-red pigment in the aerial mycelium within 9 days. The reverse of the cultures became partly or wholly pigmented as well. Cultures grown on Sabouraud maltose agar at 25° C. and then incubated at 37° C. remained white.

The fungus grew more rapidly at temperatures higher than room temperature, and grew especially well at 37° C.

The colonies of the fungus were more characteristic on Sabouraud conservation medium. They were white and developed deep, radiating furrows with

age. The peripheries of the colonies did not show any projections. The center became depressed and covered with a short, snow-white aerial mycelium which was lacking on the other parts of the colony (Fig. 4).

Development of the fungus on a medium consisting of horse manure and tap water only was rather slow, but eventually the medium became covered with snow-white mycelium.

Young cultures were characterized by the presence of numerous microconidia, either borne singly along hyphae or arranged in clusters. Many nodular organs were found in primary cultures. The most complete morphology was observed in slide cultures, 42 to 55 days old, with wort agar "Difco" using the method of Rivalier and Seydel (72); after staining, the cultures were mounted in sirop d'Apathy. The microconidia were elongated and pyriform, 2–6 μ long; they were borne singly along the hyphae or arranged in clusters. Many young (Fig. 5) and mature macroconidia (Figs. 6 and 7) were found in the slide cultures as well. The mature macroconidia were pedicellate, spindle-shaped and multicellular, measuring 7.5–20 μ by 36–90 μ. Their cell walls were quite thick and double-contoured. In some instances wart-like structures were observed on the surface of the cell wall. The macroconidia had pointed tips and most of them showed the typical bulging form of the macroconidia of the genus *Microsporum*; however, a few of them showed some similarities to those of the genus *Trichophyton*. Intercalary and terminal chlamydospores were present in great numbers. The hyphae measured 1.5–2 μ in diameter.

Five guinea pigs were shaved on the back and a crushed culture mixed with honey was streaked on the shaved surfaces. The skin of the streaked surface became red and scaly within 6 days. Scutula were found 8 days after inoculation. The inoculated fungus was recovered from these scutula by culture. The number and size of the scutula increased during the 2nd week after inoculation. In the 3rd week after inoculation a heavy tissue reaction, characterized by the formation of crusts and an excretion of a sanguinous fluid was observed in one guinea pig. The lesions of the four other animals regressed. Two guinea pigs were found free of all lesions after 25 days; the other three animals did not show any visible lesion after 32 days. The hair regrew and soon covered the infected areas.

Although we searched very carefully for infected hairs we were not able to detect a single one; not even those included in the scutula were infected.

We have no doubt that the isolated dermatophyte is identical with Quincke's α-Pilz, the former *Achorion quinckeanum*. Its morphology makes it mandatory to place the fungus in the genus *Microsporum*; therefore, it should be called *Microsporum quinckeanum* for reasons which will be discussed in detail later.

III. Epidemiological Observations on Favus of Mice in the Lower St. Lawrence Valley (Eastern Quebec)

Three field investigations were carried out in the area from which the mice had originated (Fig. 8).

PLATE I

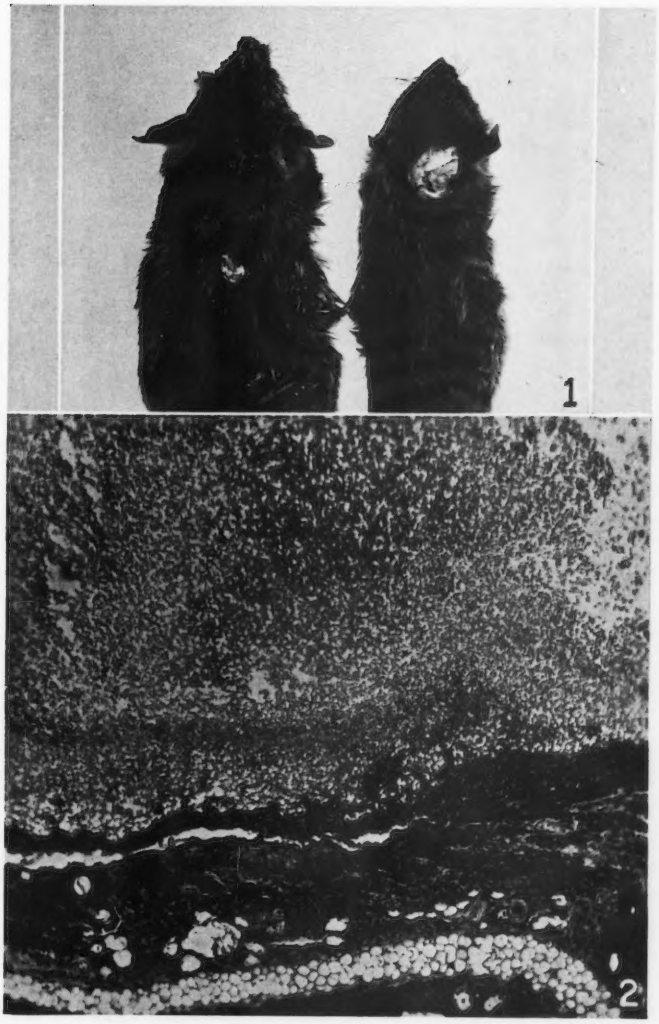

FIG. 1. Mice with scutula on the back.
FIG. 2. Section of a scutulum.

PLATE II

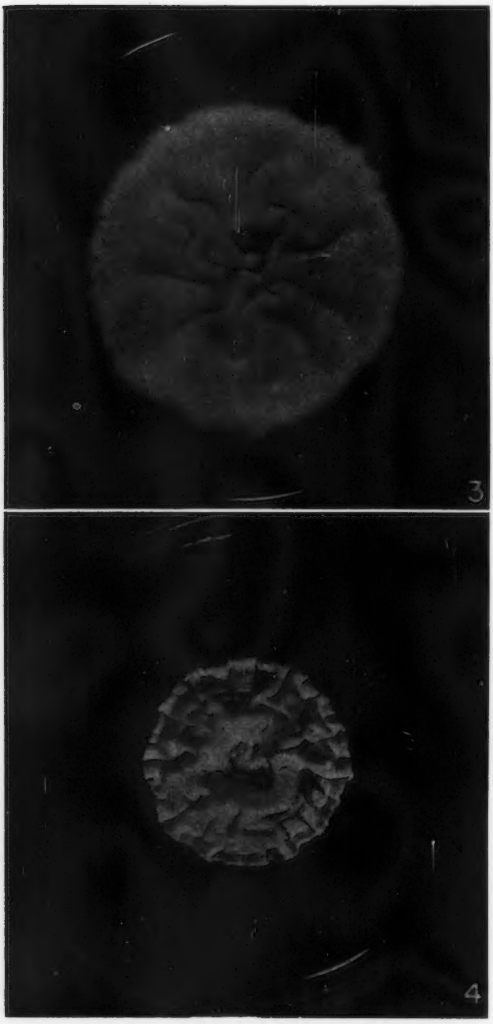

FIG. 3. Culture on Sabouraud glucose agar, grown at 25° C., 30 days old.
FIG. 4. Culture on Sabouraud conservation medium, grown at 25° C., 45 days old.

PLATE III

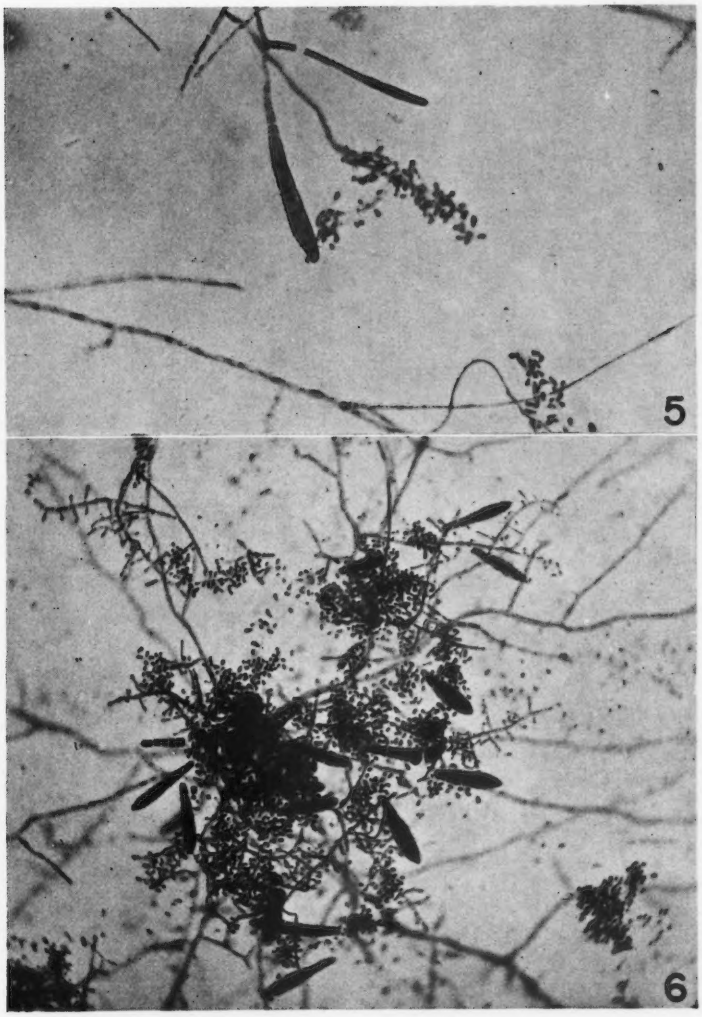

FIG. 5. Young macroconidia (slide culture).
FIG. 6. Microconidia and mature macroconidia (slide culture).

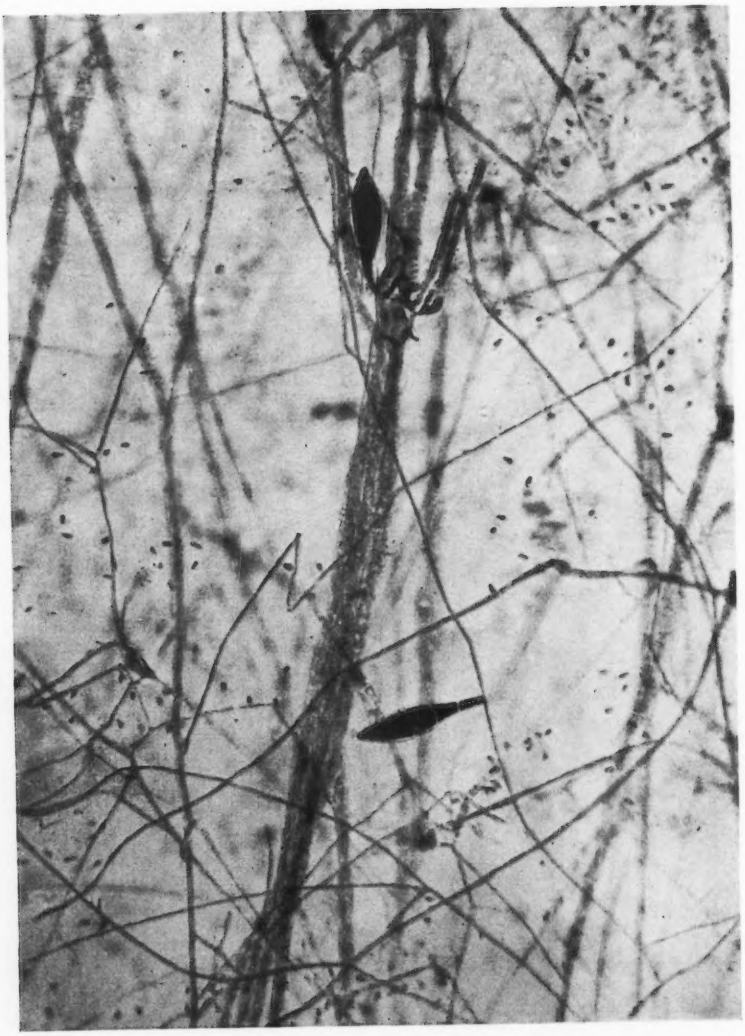

FIG. 7. Mature macroconidia (slide culture).

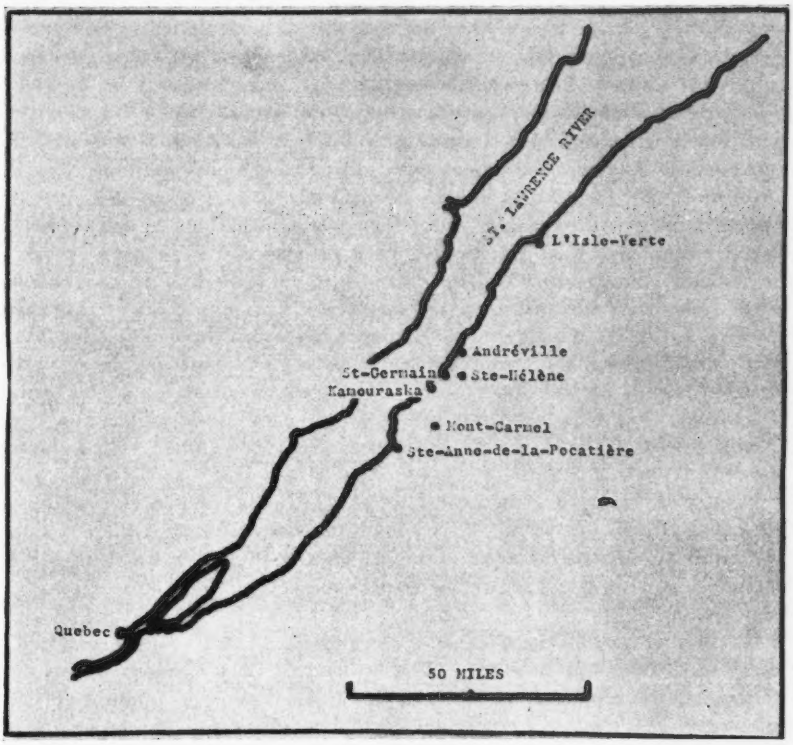

FIG. 8.

In December 1950, we went to Kamouraska where Mr. Willie LaBrie gave us four more infected mice caught by cats in his barn. These mice had favic lesions on the muzzle, ears, back, tail, and extremities. Scrapings were taken and *M. quinckeanum* was grown.

On a farm nearby, four of eight children examined were found to have small scutula on their scalps. The scutula consisted of fungal elements considerably smaller in width than those caused by *T. Schönleini.* None of the hairs examined was found to be infected. This finding held true for every specimen subsequently examined both from man and animals. *M. quinckeanum* was isolated from the scutula again. The mother of the infected children told us that she had killed a cat in August 1955 because of terrible looking skin lesions.

In St. Germain we saw a dog with scattered crusty lesions all over his body. Scutula were found on most of these lesions from which *M. quinckeanum* was grown.

In May 1956, we re-examined one of the boys in Kamouraska whom we found to have been infected the previous December. Again *M. quinckeanum*

was grown. All children of the one room school he attended were examined. A few children had scaly, very superficial lesions on their faces. However, cultures of the scrapings remained negative.

In Mount-Carmel we scraped a 12-year-old boy whose back was covered with small scutula. Scaly lesions on his face, neck, chest, and arms had persisted for 2 years. All lesions were caused by *M. quinckeanum*.

In June 1956, a cat was bought by a family in Ste.-Anne-de-la-Pocatière from a farm in Ste.-Hélène. The cat showed skin lesions 2 weeks later and subsequently she was destroyed. Part of the pelt was sent to us for mycological investigation and cultures resulted in growth of *M. quinckeanum*. Two weeks after the discovery of the cat's lesions one child of this family showed multiple, circular, sharply bordered lesions on the body. Her mother had similar lesions on the forearms, whereas the maid had large, round, elevated lesions on the upper arm. Small yellow scutula could be seen on this area. Two dogs belonging to this family showed alopecious and crusty lesions. Most of the specimens taken grew *M. quinckeanum*. The remainder showed no growth.

In August 1956, we visited the farm at which the cat had been bought in June. A girl, 17 years old, showed a well-bordered lesion on the left side of the trunk, 5 cm. in diameter, from which the fungus was cultured. The scalp was very scaly and showed an alopecious spot; however, no dermatophyte could be isolated.

On another farm in Ste.-Hélène, 2 miles away, a family epidemic caused by *M. quinckeanum* was detected. The father had crusty lesions on his scalp and body. The mother had a scutulum on her arm. A 3-month-old baby had scaly, erythematous lesions on the chin and scalp. The lesions on the scalp were contracted from the mother when the baby's head was resting on her arm during nursing. Two daughters, 7 and 11 years old, had sharply bordered, slightly raised lesions on their face, bodies, and buttocks. A dog had crusty lesions on the legs, from which the hairs could be removed easily. Two cats had already been destroyed previous to our visit.

During the same trip we examined a red fox which had been killed by boys in a field near Kamouraska 1 day before our arrival. The fox was covered with yellow crusts from which *M. quinckeanum* was cultured.

In November 1956 a patient was sent to our laboratory for mycological investigation. He was 21 years old and came from a farm in Andréville, Kamouraska County. Only a few scattered hairs were seen on his scalp; search for dermatophytes remained unsuccessful. Both eyes were the seat of chronic inflammation and the interior of the eyes was disorganized, but the etiology of this process has not yet been elucidated. Six fingernails were found to be infected. *M. quinckeanum* was grown from the nails as well as from a lesion on the right ankle.

Scaly, very superficial lesions on the faces and knees of two boys living on a farm in L'Isle Verte, County Rivière-du-Loup, were observed by the local health officer in June 1956. We scraped these two boys 2 months later and grew *M. quinckeanum*.

After these observations there seems to be no doubt that favus of mice is endemic in the lower St. Lawrence Valley, especially in the Kamouraska County. Mice represent the reservoir of the dermatophyte which is transmitted by cats and dogs to man. Wild animals may also be involved.

IV. Discussion

A lively discussion accompanied by strong criticism was stirred up by Quincke's publications (67, 68). Although he had to admit that both the α- and γ-Pilz might cause favus of the smooth skin, he remained adamant as to the validity of these two dermatophytes as being different species (69). Finally, Quincke's findings, especially the validity of the α-Pilz as a distinct species, had to be accepted, but the unfounded criticism of Quincke's work continued. Sabouraud (73, 74) even went so far as to state "à mon avis Quincke avait cultivé une impureté de la peau pour un favus, et l'*Achorion Quinckeanum* appartient à Bodin, non à Quincke, mais peu importe". Bloch (10) has already pointed out how unfounded Sabouraud's criticism was.

Contrary to Bodin's clear statement (11) Vanbreuseghem (92) recently maintained that Bodin's *Achorion quinckeanum* was not identical with Quincke's α-Pilz for three reasons which do not appear valid to us. First, he pointed out the differences in the pigmentation of the strains. We have seen such differences in our strains of *M. quinckeanum* as well. They occur also in other dermatophytes, e.g. *Trichophyton rubrum*. Secondly, Bodin did not describe macroconidia which Quincke had found and reported. It appears that Bodin examined microscopically only cultures grown on solid media. We, too, have never found macroconidia in cultures grown on solid media. Sabouraud (73) described the formation of macroconidia in hanging drops as did other authors (10, 22, 77, 85, 94). Quincke (67) already pointed out that special conditions (e.g. exhaustion of the medium and sufficient oxygen supply) must prevail to ensure the formation of macroconidia. A third point raised by Vanbreuseghem as being different in Quincke's and Bodin's papers is the invasion of hair, which was never observed by Quincke. Bodin remarked that the hairs of experimentally infected guinea pigs "n'étaient que très peu atteints par le parasite". Vanbreuseghem observed an invasion of experimentally infected guinea pigs as well. We have never seen an invasion of hairs in spontaneous infections (mice, cats, dogs, fox, and men) and in experimental infections (guinea pigs) and believe that these discrepancies do not have any great importance.

The taxonomic position of this dermatophyte has never been decided definitely. Quincke (67, 68, 69) never gave a binominal name to his α-Pilz. Bodin (11) believed that the dermatophyte was more closely related to species of the genera *Trichophyton* and *Microsporum* than to the agent of human favus known as *Achorion Schönleini* at that time. However, he did not want to make any decision as to its taxonomic position until the morphology of *A. quinckeanum* was better known. Sabouraud (73) placed all dermatophytes causing the clinical entity of favus in the genus *Achorion* although he was well aware that *Achorion gypseum* for example, because of its morphological

features, should find its place among the *Microsporum* species of animal origin. Further studies of the morphology of the dermatophytes have invalidated Sabouraud's equation (favus=*Achorion* (46)) and the genus *Achorion* became obsolete. Hence the taxonomic position of *Achorion quinckeanum* became "incertae sedis".

Langeron and Milochevitch (47) studied one strain of this dermatophyte which they obtained from the Sabouraud collection. Although they did not find any macroconidia they placed the fungus in the genus *Sabouraudites* in which they united the *Microsporum* species with those former *Achorion* species presenting morphological features of the genus *Microsporum*. Dodge (21) called Quincke's α-Pilz *Achorion muris*. Emmons labelled it *Microsporum quinckeanum* in 1934 (26) and *Trichophyton quinckeanum* in 1947 (84). Vanbreuseghem (92) placed the etiologic agent of mouse favus in the genus *Trichophyton*.

As stated before, the causative organisms of favus of mice (Quincke's α-Pilz, *A. quinckeanum*) belongs to the genus *Microsporum* and, therefore, becomes *M. quinckeanum* for the following reasons:

1. The great majority of the macroconidia are pedicellate, and spindle-shaped with pointed tips. They have been seen by other authors before although their published reproductions are not always too clear (10, 22, 67, 77, 85, 94).
2. The microconidia are elongate and similar to those seen in other *Microsporum* species.
3. The primary cultures grow quickly, and in the beginning are as flat as those of other *Microsporum* species.
4. When slide cultures of *M. quinckeanum* were mounted in Canada balsam, the macroconidia showed a shrinkage similar to those of *M. gypseum*, a phenomenon we have observed in these two species only.
   The fact that both *M. quinckeanum* and *M. gypseum* have never been found to affect hairs in cases of spontaneous infections may also be mentioned in this connection.

The combination *Microsporum quinckeanum* (Zopf) Guiart et Grigorakis* is hereby established.

Dr. E. Rivalier (Laboratoire Sabouraud, Hôpital St. Louis, Paris), to whom we sent microphotographs of our slide culture, agreed with our opinion as to the taxonomic position of the agent of mouse favus.

The literature on mouse favus in man and animals is widely spread. It is very often hard to evaluate whether the authors were dealing with inoculations of mice and man by *M. quinckeanum* or by other dermatophytes. In other instances, the description of the dermatophyte leaves no doubt that the isolated fungus was not *Microsporum quinckeanum* (30, 83).

M. quinckeanum was isolated in Belgium (92), Czechoslovakia (59), Denmark (5), France (11, 12, 58, 70), Germany (7, 28, 29, 36, 39, 41, 42, 43, 44, 65, 67, 68, 75, 76, 77, 80, 85, 94, 95), Great Britain (1, 2, 3, 23, 50, 51, 52,

* J. Guiart and L. Grigorakis, La classification botanique des champignons des teignes. Lyon Médical, **141**, 369–378 (1928).

53, 54, 89, 93), Holland (6, 20, 60, 66), Hungary (4, 57), Italy (16, 90), Russia (38, 40, 55, 87), and Switzerland (10, 22). There are also reports on favus of mice from North Africa (14) and Australia (19, 48, 61).

A search of the literature revealed three reports of favus of mice in North America. Greenbaum (37) reported from Philadelphia the isolation of *M. quinckeanum* from the lower eye-lid of a 10-year-old boy and Shaw and Wampler (79) found favus in mice in Virginia. Whether *M. quinckeanum* was actually the causative organism in these two instances is hard to ascertain. More recently, Sisk, Woolridge, and Lamb (83) reported two isolations from St. Louis. However, Dr. L. K. Georg (34), who examined these two cultures, identified them as *T. Schönleini*.

Most human infections reported were isolated cases with lesions involving the smooth skin only. These usually showed small scutula although in some instances the lesions remained very superficial (10, 44), observations which induced Bloch (10) to speak of "favus sine scutulis". Wandel (94), a pupil of Quincke, most probably was the first who observed the very rare infection of the scalp by *M. quinckeanum*. Beintema (6) reported a case of kerion formation.

Epidemics were reported by several authors (19, 20, 28, 29, 48, 61, 66). Fischer (28, 29) described an outbreak among millinery workers. Many mice were found on the premises and cats displaying scutula were thought to have transmitted the disease to the workers. During the First World War an epidemic occurred among Dutch soldiers stationed in a school infested with mice (20). In Australia, at certain times and associated with large wheat stacks, there have been plagues of mice; some of them have been found to be affected with favus (*M. quinckeanum*). Through the handling of bags with which these mice have been in contact many men became infected and *M. quinckeanum* was cultivated on various occasions from men and mice. Mice died from the disease: the growth in some cases entered by the orbit; in other cases it caused destruction of the limbs, the tail, and in some cases, it penetrated through the superior maxilla. Sporadic cases unassociated with the handling of wheat are found in Australia (19, 48, 61), but are rare.

The transmission of the disease from mice (2, 3, 17, 20, 50, 51, 65, 66, 89, 94), cats (7, 28, 29, 95), dogs (77), and horses (36, 85) to man has been reported. There seems to be no doubt that cats and dogs who acquire the disease from mice, its normal hosts, transmit it to man.

Acknowledgments

These investigations were initiated by the fine observations of a great naturalist, Mr. Willie LaBrie, Kamouraska, P.Q. The first specimen (infected ear of a mouse) was received from Dr. P. J. G. Plummer, Animal Diseases Research Institute, Hull, P.Q. In his field investigations the author enjoyed the full co-operation of Dr. A.-R. Foley, Ministère de la Santé, Quebec; Dr. R. Lizotte, Dr. G. Martineau, and Dr. E. Richard, Unité Sanitaire, Ste.-Anne-de-la-Pocatière, P.Q.; and Dr. V. Ratté, Unité Sanitaire, Rivière-du-Loup, P.Q. We wish to express our sincere gratitude to all of them.

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THE MORPHOLOGY OF A SPECIES OF THE BACTERIAL GENUS CYTOPHAGA WINOG. IN CULTURE[1]

P. H. H. GRAY

Abstract

A culture of the cellulolytic bacterial organism, *Sporocytophaga myxococcoides* (A.T.C.C. No. 10010), has been studied. In or on media containing cellulose, the primary effective cells are long and acuminate; in old cultures a large proportion of them become spherical or spheroid and are bound together in mucilage, in which new spheroid cells develop. In or on media containing cellobiose, glucose, or mannose as the source of carbon, the primary effective cells resemble those on cellulose media, but the resting cell is a spherical granule, its diameter not more than twice the width of the acuminate cell; the spheroid cells do not become aggregated in the mucilage, and do not physically resemble the so-called microcysts except in shape.

The binomial *Cytophaga hutchinsoni* was applied by Winogradsky (11) to the bacterial microorganism originally named *Spirochaeta cytophaga* by Hutchinson and Clayton, who first isolated and studied it in pure culture (3). Its chief distinguishing characters were: physiological (its ability to utilize, apparently, only cellulose as a source of carbon) and morphological (having, in young cultures, thin curved or twisted filamentous acuminate cells from which developed oval and spheroid cells ('sporoids') from which, in turn, new filamentous cells emerged). Hutchinson and Clayton appear to have made the untested assumption that the new filamentous cells arose from the sporoids; in so doing they probably mistook the juxtaposition of the two forms for evidence that the filament emerged from the sporoid. They also failed to remark upon the apparent development of sporoid from sporoid, which is clearly observable in their photographs (Plate II, Fig. 3 and Plate III, Fig. 4).

Krzemieniewska (5) attempted to show that the cells passed through a cycle of morphological changes identical with that suggested by Hutchinson and Clayton, and introduced the term microcyst to replace sporoid. Neither Winogradsky not Krzemieniewska, however, studied pure cultures. Stanier has reviewed the literature up to 1942 (7). In 1942 also (8) he reported that *Cytophaga* cultures could grow in media with glucose instead of cellulose as the source of carbon, provided that the glucose solution was sterilized by filtration through a Seitz filter and not by heat. He did not state if the morphology of *Cytophaga* was altered. Fåhraeus also reported (1) that a microcystogenous strain of *Cytophaga*, isolated from a garden soil, could utilize glucose, provided that the glucose solution was autoclaved separately from the salts medium. He did not describe the morphology of the cells in those cultures. A more recent paper by Sijpesteijn and Fåhraeus (6) suggests

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(Plate I, Fig. 5) that most of the cell forms described by Hutchinson and Clayton developed in 5 days in a 1% glucose liquid medium and in 8 days (Plate I, Fig. 8) on a 0.1% glucose agar medium. Those cultures had been tested for the absence of associated bacteria by transfer to non-cellulosic media. The authors did not describe a sequence of changes in cell morphology. The senior of those two authors had previously discovered (in 1942, unpublished results (see reference 6)) that the strains isolated "were able to grow, though badly" in media autoclaved with 0.1% glucose therein; and more recently (6) that they would grow well at that concentration provided the pH of the media before autoclaving did not exceed 7.0. The effects of such conditions on cell morphology were not stated.

The apparent existence of microcyst-forming and non-microcyst-forming strains of the cellulolytic *Cytophaga* species led Stanier to separate the two forms taxonomically, the former group being named *Sporocytophaga*. The division was based on the nature of cell development, but only in or on media containing cellulose. It is unfortunate that the definition of the genus *Cytophaga* was based on studies with impure cultures, but the agreement of its characters with those recorded by Hutchinson and Clayton render it an acceptable term to replace *Spirochaeta*.

Some workers, notably Jensen (4), state that the microcyst-forming strains may lose their ability to do so. It is possible that they could not form microcysts in pure culture if cultivated in or on media without cellulose, and at reasonably low temperatures, nearer to those at which they develop naturally. Stanier states (7) that "the gradual diminution of microcyst formation in pure cultures (of *Sporocytophaga*) . . . is a very marked phenomenon. Only in impure or in recently purified strains of *S. myxococcoides* can one observe the typical . . . "Mikrokokkenschleim" around cellulose fibers . . .".

It is usually difficult to isolate these bacteria in pure culture, not only because of the numerous other species of bacteria that accompany them in the process of cellulose decomposition, but also because of the bacterial gum that increases in quantity as the culture ages and serves to entrap other kinds of bacteria. Inoculation of primary cultures into nutrient broth or onto nutrient agar, containing beef extract, is not at all times a safe basis for assuming that lack of growth therein or thereon is proof that there are no associated bacteria with the *Cytophaga* for there are some bacteria that grow only slowly in media containing beef extract, and some that do not grow; on several occasions the author has observed a contaminant *Micrococcus* species which did not grow after transfer, and on one occasion a *Bacillus* which appeared to have destroyed the *Cytophaga* but was itself destroyed later by the rejuvenated *Cytophaga* in the same culture.

Hutchinson and Clayton were satisfied that they had obtained a pure culture after they had diluted to a high degree a young culture in which the sporoid was not visible, but the sporoid as well as the 'intermediate forms' developed in cellulose media inoculated from the cultures of purely filamentous cells. Recently, in this laboratory, Yaphe (12) prepared many series of

transfer cultures to media containing cellulose, from 1-, 2-, 3-, 4-, and 6-day cultures of *Sporocytophaga myxococcoides* A.T.C.C. No. 10010, and found that the sporoids eventually developed in all of the transfer cultures.

No worker has yet attempted to determine the proportions of the various cell forms as they appear in sequence, from the early logarithmic growth phase, when presumably the culture consists of 100% filamentous cells, to the old culture containing, also presumably, 100% resting cells (sporoids). The developmental 'cycle', e.g. that reported by Grace (2), seems to have been built up from observations of the kinds of cells seen in apparent sequence by inference and not by enumeration of the forms in a progression similar to that found in the progenies of differentiating meristematic tissue cells of a growing root.

Studies on *S. myxococcoides* A.T.C.C. No. 10010 were continued in this laboratory in November 1953 with the following aims:

(1) to determine the most suitable media and the best conditions for the growth of a pure culture;

(2) to compare the cycle of morphological changes in or on media with cellulose and other sources of carbon;

(3) to establish the sequence of those changes on a quantitative basis, similar to that developed by Thornton and Gangulee in studies on the nodule bacteria, *B. radicicola* (10).

This report covers chiefly the search for suitable media and a study of the development of cell forms in media containing different sources of carbon, nitrogen, and other requirements (1 and 2 above). All media were sterilized in the autoclave. The medium used for most of the earlier work was a mineral salts basic medium containing the following salts in % (w/v): K_2HPO_4 0.1, $MgSO_4.7H_2O$ 0.02, NaCl 0.01, $CaCl_2$ 0.01, $FeCl_3.6H_2O$ 0.002, with KNO_3 0.2 or $NaNO_3$ 0.17, and with filter paper (usually Whatman No. 41) as the source of carbon. After several months of culturing at 26–28° C., and checking for absence of contaminants and associated bacteria, and after re-culturing from well-isolated colonies on agars containing glucose, it seemed to be reasonably safe to assume that the culture was a pure cellulolytic *Cytophaga*, with characters conforming to those described by Hutchinson and Clayton.

It was necessary to ensure that the cultures to be studied contained, at the start, only the sinuous acuminate (fusiform) cells. Advantage was taken of the fact that the acuminate cells are able to move through liquid films when near or in contact with cellulose fibrils. It was assumed that if the movement were continuous and radially progressive from the point of inoculation, acuminate cells only should be found at some distance from it, and that only a few should be visible in several fields of a stained film prepared from the paper at that hypothetical position, within a calculable period of time. Some of the cultures made to test for associated or infecting bacteria were prepared by inoculating small circular disks ('confetti') of filter paper, 6 mm. diameter, made by means of a paper-punch; each sterile disk was picked up by means of a loopful of sterile water and thus easily laid on the agar. After it had been found that the various cell forms of *S. myxococcoides*

were visible in heat-fixed films stained with crystal violet,[2] the whole disk was transferred to Bacto nutrient agar;[3] if there was no growth on the agar and the cells seen in the films appeared to be only those similar to the various forms described and illustrated by Hutchinson and Clayton, the culture was registered as suitable for further study. A sterile disk was then placed on the surface of mineral salts nitrate agar in a Petri plate and inoculated lightly with culture on a platinum wire; sterile disks were then placed in the form of a tower of four or more disks on top of that disk. Single, separate, disks on agar in another plate were inoculated at the same time.

After 5 days the disks, not yet visibly yellow, were aseptically removed, separately, from the pile, placed for a few seconds on a sterile microscope slide, and then placed in another plate of agar. The films on the slide were fixed with heat and stained with crystal violet. The acuminate cells only were present in all of the films.

The bottom disk had been placed on agar, and the upper disks each on a sterile disk on the agar. After 24 hours all of the disks were yellow and each contained only the acuminate cells. After another 48 hours films were again prepared from the disks; spheroid[4] cells were present in all of the films, in the proportion of about one-third spheroid to two-thirds acuminate cells. It was noted that there were no 'intermediate forms' present in the films. Suspensions prepared from these disks, with distilled water, were inoculated onto fresh disks on slopes of Bacto nutrient agar and salts nitrate agar to test for purity of the disk-cultures and their viability.

It was decided from these tests that the culture was free from associated or infecting bacteria or could easily be made free by these simple precautions, and that the spheroid cells could be found as a normal stage in the sequence of development of cell forms in cultures on filter paper.

Amongst the media used for receiving the inoculated disks of filter paper were the following, in addition to the two mentioned above: Bacto tryptone glucose extract agar, both according to directions on the label and diluted 1:2 or 1:4 with water and with agar added to make a 1% gel; and a mineral salts agar (1.0%) or fluid medium composed of tryptone, glucose (or cellulose), starch, yeast extract, calcium carbonate, all 0.1%. The bacteria grew vigorously on these media without the paper disks, and, moreover, grew only as the acuminate rod. Cellobiose was found to be a superior substitute for glucose, in that the young cells were larger and stained more intensely with crystal violet and survived longer. Sterilizing the glucose or cellobiose separately from the salts did not affect the sequence of cell forms. When cellulose replaced the glucose or cellobiose, however, the spheroids developed, but without 'intermediate' forms; the spheroids developed also when cellulose powder[5] was used in place of the paper.

[2]Made by Difco Laboratories Incorporated, Detroit, Michigan, U.S.A.
[3]Made by Difco Laboratories Incorporated, Detroit, Michigan, U.S.A.
[4]In the present article the terms 'sporoid' and 'microcyst' have given place to the descriptive term 'spheroid', in order to remove any connotation with a hypothetical physiological implication.
[5]That prepared by W. & R. Balston, Ltd., as Genuine Whatman Cellulose Powder, or that obtainable as Alphacel (Nutritional Biochemicals Corporation).

The forms of vegetative cells observed in cultures grown in or on media containing cellobiose, or glucose, at 26–28° C. were of three kinds: the primary vegetative stage, an acuminate undulating spirochaetoid form, as shown by Hutchinson and Clayton in their Fig. 1 (second diagram from the left), but staining uniformly with either aqueous crystal violet or carbol erythrosin; the second vegetative stage, an acuminate or truncate spirochaetoid with the cytoplasm staining in a shorter lengthwise band; the granule spirochaetoid, in which the acuminate cell contained a deeply stained spherical globose granule, often twice the width of the containing cell; these were succeeded by the final, or resting, stage, which was a free globose granule. This time-sequence of forms occurring in films of increasing ages is shown diagrammatically in Fig. 1.

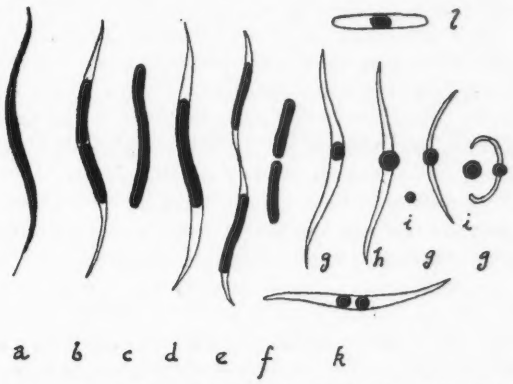

FIG. 1. The most frequently seen forms of cells developed by *Cytophaga* in non-cellulosic media, up to 30 days at 26–28° C. *a–d–b–e* and *d–e–c–f* may be regarded as the forms developing in those sequences leading to the resting stage free granule *i*; *k* may be a form succeeding *b* or *e*; form *l* has rarely been seen.

The primary acuminate form of cell appears as shown only in fixed and stained films. By dark-field illumination these cells appear truncated, the chromatin in 5-day or older cultures being grouped at the two ends. The cells have the same acuminate shape alive (in wet mounts under direct light) as when fixed (either by gentle heat or by osmic acid fumes); none of the films of the many retained contained "distorted ... twisted spiral rods" stated by Grace (2) to have been described by Hutchinson and Clayton. (That statement appears to be a distortion of a statement by the original authors.) The various forms of cells illustrated in Fig. 1 can be seen in cultures up to 1 month old. The best demonstration of the "sequence of forms" described here has been found in films made from cellobiose asparagine salts fluid medium.

The 'microcyst' has never yet developed in non-cellulosic media. Cells inoculated from a spheroid-free culture in or on cellobiose or glucose into a medium containing filter paper or cellulose powder develop into cultures containing the so-called microcyst relatively early, as soon as a faint yellow

appears on the paper or powder, whatever ingredients the basic medium contains. It has been noted that when films are prepared from a fluid medium containing a strip of filter paper, after the culture has been shaken gently to loosen the disintegrating fibers, only a few spheroids will be found and those usually in masses of what may be gummy bacterial debris, whereas if the film is made with material more forcibly removed from the paper, a much greater proportion of the cells are spheroids (cf. Hutchinson and Clayton (3), Plate II, Fig. 6 and Stapp and Bortels (9), Plate II, Fig. 12). In some films made from filter paper or powder cultures the spheroids can be seen in pairs or groups of connected globular cells, giving the appearance of such cells dividing. This was illustrated by photograph, but not commented on, by Hutchinson and Clayton and by Stapp and Bortels. Such agglomerations may consist of 'cells' of the same dimensions or of different dimensions. These 'cells' are much larger than the resting stage granule in cellulose-free media; and they differ amongst themselves in that only a few in a film, treated first with *N* or 0.5 *N* HCl and stained with crystal violet, appear to contain a deeply staining granule. It may be possible to demonstrate that in media containing cellulose the 'sporoid' or 'microcyst' is identical with the ultimate resting-cell, or granule, developed in or on the cellulose-free media but has, by reason of its different chemical and physical environment, served as an attraction point for the aggregation of successive layers of capsular (polysaccharide) gum, containing mucin, which is capable of absorbing considerable amounts of the basic dye. This is strongly suggested in films treated with hot 0.5 *N* HCl, in which the granule is usually well defined within the spheroid. Under dark-field illumination the spheroid appears either as a stout ovate short rod with a dark center and a bright lunate area at each end, or as a bright ring; both forms are of a diameter equal to but not as much as twice the diameter of the acuminate rod.

Acknowledgments

Studies on *Spirochaeta cytophaga* were made in 1924–1928 in the James Mason Laboratory at the Rothamsted Experimental Station, Harpenden, England, under the direction of Dr. H. G. Thornton, to whom the author is much indebted for help in those early studies. During those studies attempts to observe the germination of new acuminate cells from the 'sporoids' isolated as 'single cells' were abandoned because it soon became obvious that it would be impossible to be certain that there were no small acuminate cells, invisible by direct light, attached to the mucilaginous envelope of the 'sporoid'. An apparently 'sporoid-free' culture which developed after 4 years of rest in autoclaved soil was unfortunately lost by misadventure.

The author is grateful also to Mr. G. V. Jacks, Director of the Commonwealth Bureau of Soil Science, for providing a translation of part of the paper by Stapp and Bortels, and photostats of papers by Mdme. Krzemieniewska; and to Professor R. H. Wallace, his long-suffering colleague, who at all times cheerfully undertook to confirm much of the evidence provided by the culture media and the microscope.

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SOME OBSERVATIONS ON THE METABOLISM OF STREPTOMYCES SCABIES[1]

R. J. Douglas and C. L. San Clemente[2]

Abstract

In the presence of the dye 2,6-dichlorophenol indophenol crude extracts of alumina-ground or of sonically disrupted mycelium of *Streptomyces scabies* catalyzed dehydrogenase reactions with the substrates succinate, citrate, malate, and glutamic acid. These extracts or mycelial homogenates formed glutamic acid in transaminase reactions with α-ketoglutarate and the amino acids aspartic, leucine, and valine. Other amino acids, lysine, threonine, glycine, histidine, and tyrosine, failed to act as amino donors. No evidence could be obtained for direct transamination with α-ketoglutarate and glutamine or asparagine; with the latter, analyses suggested that glutamic acid formation occurred by preliminary deamination and subsequent transamination from the aspartic acid produced.

Introduction

The metabolism of organisms of the genus *Streptomyces* has been the subject of a number of recent investigations (4, 5, 9, 12); most of these have been concerned with organisms involved in antibiotic production (14). Studies in this laboratory have centered on the metabolism of the potato scab organism, *Streptomyces scabies*, and have developed with a view towards a possible explanation of the pathogenic activities of the organism on a biochemical basis. In a previous report (7) the respiratory activities of mycelial homogenates were described; the present work is an extension of this and deals with some dehydrogenase and transaminase activities of mycelial extracts.

Materials and Methods

Mycelium was grown in peptonized milk with glucose (0.17%) or in nutrient broth plus 1% glucose. The cultures used, conditions of incubation, and methods of harvesting have been described (7). Washed mycelium was ground with an equal volume of alumina for 10 minutes in a glass homogenizer chilled in an ice bath and extracted with phosphate buffer (pH 7.0). Alumina and cell debris were removed by centrifugation at 1600 × *g* for 10 minutes and the crude extracts maintained in an ice-water bath until used. Nitrogen was determined by the micro-Kjeldahl method. In a few experiments extracts were prepared following mycelial disruption in a 10 kc. Raytheon oscillator; at peak output the maximum nitrogen was released in 4–7 minutes.

Dehydrogenase Experiments

Alumina extracts showed only slight oxygen uptake manometrically even when fortified with ATP, DPN, and Mg ions. The dye 2,6-dichlorophenol indophenol (DCPP) was chosen to replace oxygen as an electron acceptor;

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the low toxicity, high extinction coefficient, and negligible autooxidation characteristic of DCPP have been described by Repaske (13) and Haas (10). Reactions were carried out as follows: buffer, supplements, substrate (or water), and cell extract were added to 12 × 75 mm. cuvettes, mixed by inversion, and the optical density at 600 mμ set at zero. A solution of DCPP was then added (final concentration 0.002 or 0.0013%), the tube contents mixed and the OD_{600} determined immediately and at 30 second intervals for a period of 5 minutes. Contents of the cuvettes were mixed following each reading. Endogenous controls contained all additions except substrate.

Transaminase Experiments

These reactions were carried out in stoppered tubes in a water bath at 37° C. Reaction mixtures and controls were prepared according to the following plan:

Tube	Amino acid	Keto acid	Water	Extract
1	+	+	−	+
2	+	−	+	+
3	−	+	+	+
4	−	−	++	+
5	+	+	−	Boiled

Amino and keto acids were present at 0.05 *M* in a 0.025 *M* phosphate buffer pH 7.0. Each milliliter of reaction mixture contained 0.25 ml. of crude extract. At the end of the incubation period the reaction was stopped by boiling the mixture for 5 minutes or steaming it for 10 minutes. Solutions were chromatographed in water-saturated phenol (26° C.) on Whatman No. 4 paper and dried at room temperature before a fan. The sheets were sprayed with 0.1% (w/v) ethanolic ninhydrin and the color allowed to develop at room temperature or hastened by a short exposure to flowing steam.

Semiquantitative results were obtained by eluting standard sized areas containing the amino acid with 50% (v/v) ethanol and determining the optical density at 570 mμ in a Coleman junior spectrophotometer against a blank obtained by eluting an equal area of paper from the same sheet This was shown to be a suitable quantitative method when comparisons were restricted to analyses on a single sheet. In some experiments glutamic acid formation was measured with the decarboxylase present in acetone-dried *E. coli* ATCC 4157. Transamination was indicated where the net glutamic acid formed in the complete mixture (tube 1-4) was greater than the sum of the amounts of glutamic acid formed when the reactants were individually omitted [(tube 2-4) + (tube 3-4)].

Valine and threonine were racemic mixtures; the other amino acids were L- forms. The amino and keto acids and the preparations of ATP and DPN were obtained from Nutritional Biochemical Corp., non-nitrogenous acids were obtained from British Drug Houses Ltd., and pyridoxal phosphate from Bios Labs.

Results

Dehydrogenase activity was demonstrated with alumina extracts, with the substrates citrate, malate, fumarate, and succinate, and with glutamic acid. The activity of these extracts, as determined by the change in optical density during the initial stages of the reaction (8, 13), varied considerably from extract to extract but was proportional to the amount of extract used. Control determinations always showed a high endogenous activity although this could be materially reduced by dialysis. The extracts were deficient in pyridine nucleotide as evidenced by the stimulatory action of additions of this substance (Fig. 1). Citrate dehydrogenation showed greatest stimulation by TPN whereas DPN was more effective with glutamic acid. Dye reduction with malate required DPN. With some extracts α-ketoglutarate dehydrogenation was observed, but this activity was variable and of a lower order than observed with glutamate.

In DCPP reduction experiments with crude sonic extracts cyanide (10 micromoles (μM.)) was shown to exert a pronounced stimulatory influence. This probably resulted from an inhibition of the cytochrome system (11) since these extracts were active in manometric assays.

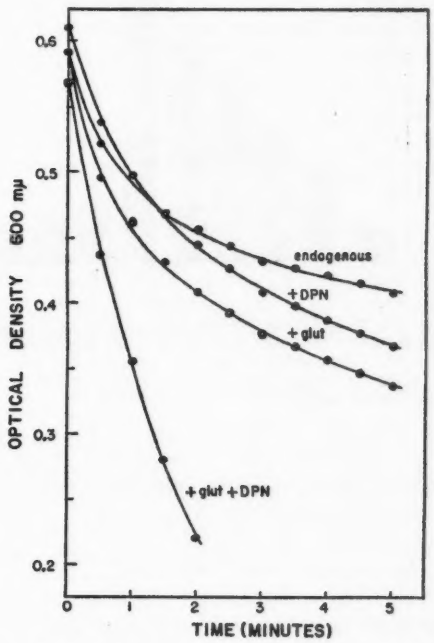

FIG. 1. Reduction of 2, 6-dichlorophenol indophenol endogenously and in the presence of glutamate by 0.5 ml. of crude extract (1.4 mg. N/ml.) and stimulation by DPN (500 μg./3 ml. reaction mixture).

Alumina extracts catalyzed glutamic acid synthesis from α-ketoglutarate and the amino acids aspartic, leucine, and valine. Some extracts were able to utilize alanine for glutamic acid synthesis but this activity was variable. Negligible activity was observed with the amino acids glycine, threonine, histidine, lysine, methionine, or tyrosine. The most active amino donor was aspartic; with this amino acid glutamic acid was synthesized at rates up to 10μM./mg. N/hour. Results of a typical experiment are shown in Fig. 2. The activities observed with leucine and valine were about 40% of the rate with aspartic acid. It was not possible to increase the range or extent of transaminase activity by the addition of pyridoxal hydrochloride plus ATP or pyridoxal phosphate. Cell homogenates and acetone-dried mycelium also catalyzed the reactions described above.

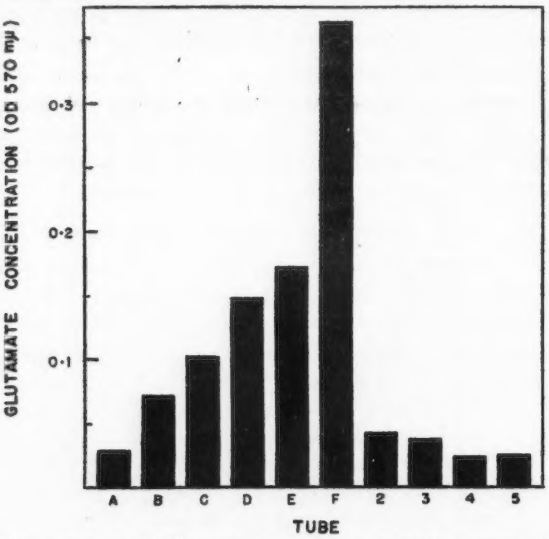

FIG. 2. Time course of the glutamic–aspartic transaminase reaction. Bars A to F represent relative glutamate concentrations in the complete reaction mixture sampled at 0, 15, 30, 45, 60, and 120 minutes respectively. Bars 2, 3, 4, and 5 represent glutamate in the controls (see methods) sampled at 120 minutes. Each tube contained 0.3 mg. extract N/ml. reaction mixture.

These extracts contained considerable amounts of amino acids; by the use of added internal standards ninhydrin-positive materials were observed whose R_F values were identical with those of glutamic, alanine, and leucine. Other, less well-defined, materials were also present. In most cases the control containing only α-ketoglutarate showed an increased glutamic acid concentration; similar observations have been made by Schepartz (15) and by Bigger-Gehring (1). This endogenous glutamic acid formation made it imperative to carry the controls previously described in order to assess net transaminase activity.

With oxalacetate as an NH_2-acceptor aspartic acid formation could be shown only with glutamic as the donor amino acid. It was not possible to

demonstrate alanine formation from pyruvate with any of the amino acids mentioned above.

An attempt was made to show glutamic acid synthesis from α-ketoglutarate and the amides asparagine and glutamine but with the latter there was no evidence of transaminase activity by the criterion previously described. Asparagine supported glutamic acid synthesis but the control lacking keto acid showed an accumulation of aspartic suggesting that the amide was first deaminated and that the aspartic formed then reacted to form glutamic acid.

Discussion

In dehydrogenase experiments the high endogenous activity made it necessary in some cases to resort to dialysis in order to demonstrate activity with added substrate. It is suggested that at least a portion of this activity is supported by glutamic acid; this compound is always present in the extracts (in amounts up to 9 μM./ml.) and is rapidly utilized by both cell homogenates (7) and alumina extracts. Glutamic acid appears in extracts obtained from mycelium of *S. scabies* grown in an asparagine–glucose–salts medium and has been reported to be present in loosely-bound form in the mycelium of *S. griseus* (9). It would be interesting to know whether this property of glutamic acid accumulation is characteristic of other *Streptomyces*.

No explanation can be advanced for the frequently noted failure of extracts to utilize α-ketoglutarate. Casida and Knight (3) working with *Penicillium chrysogenum* were able to show dehydrogenase activity on α-ketoglutarate with DCPP although manometric demonstration was not possible even with elaborate reconstitution methods. Difficulty in observing activity on this substrate has also been reported by Schatz *et al.* (14) and by Gilmour *et al.* (9) for other *Streptomyces*. The previous suggestion (7) that the anomalous oxidative activity of homogenates with α-ketoglutarate was the result of permeability difficulties cannot be the whole explanation since such homogenates were active in transaminations involving this substrate.

With alumina extracts oxygen uptake could not be demonstrated consistently; methylene blue did not satisfy the terminal electron transfer requirements. The addition of phenazine-methosulphate (5, 16), however, considerably enhanced activity. Experiments using sonic extracts and this electron carrier are at present underway.

The limited scope of transaminase activities observed is in striking contrast to that recorded for some other organisms and may be a reflection of incomplete extraction or inactivation of the enzymes concerned (1, 2). The high order of activity of the glutamic–aspartic system, however, is consistent with that noted with other tissues (6).

Acknowledgments

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OBSERVATIONS ON THE EFFECT OF AMINO ACIDS ON THE GROWTH INITIATION OF RHIZOBIUM MELILOTI, WITH SPECIAL REFERENCE TO THE SYNTHESIS OF ALANINE FROM PYRUVATE AND AMMONIUM IONS[1]

D. C. JORDAN

Abstract

Washed cells of *Rhizobium meliloti* were capable of forming pyruvate from glucose and, in addition, washed cells and sonic extracts possessed a reversible alanine dehydrogenase, capable of forming alanine from pyruvate and NH_4^+. This synthesis of alanine was optimum at an alkaline pH and at a substrate concentration of 0.025 *M* and was stimulated by diphosphopyridine nucleotide but not by triphosphopyridine nucleotide. Sonic extracts in the presence of NH_4^+ also formed glutamate from α-ketoglutarate and aspartate from fumarate. Nevertheless, washed cells did not initiate growth in a 0.25% carbohydrate medium, containing NH_4Cl, unless amino acids were present. These requisite acids either could be supplied in the medium, or the cells could be forced to synthesize them by addition to the medium of increased levels of certain compounds, such as 0.9% glucose, from which NH_4^+-accepting compounds could be produced. If the stimulative effect of amino acids in low-carbohydrate media were a result of an increase in the accumulation of such NH_4^+-acceptors such an accumulation did not apparently result from increased carbohydrate oxidation or decreased "oxidative" assimilation, since NH_4^+ and α-dinitrophenol, which do not initiate growth, were more active, respectively, in these two latter aspects than the amino acid (histidine) tested. On the basis of several considerations it is hypothesized that the primary effect of the growth-initiating amino acid may be directed toward the synthesis of a labile protein, intimately connected with growth, which is destroyed in resting cells.

Introduction

Washed cells of *Rhizobium meliloti*, strain R_{21}, will not initiate growth in a chemically-defined medium containing ammonium chloride and 0.25% glucose or sucrose, unless any one of a large number of amino acids is present (9). Once growth has begun the ammonium chloride is used as an additional source of nitrogen (13). Growth can also be initiated, however, if the amino acid is replaced by any one of several non-nitrogenous carboxylic acids of importance in the tricarboxylic acid cycle (13), thus indicating that resting cells of this organism can synthesize amino acids from ammonia, but only when the concentration of certain cellular intermediates is at a particular level. Enzymes responsible for such syntheses are well known and at least one such enzyme, L-glutamic acid dehydrogenase, is known to be present in this particular bacterium (13).

Studies in this laboratory (11) have indicated that this initiating effect of amino acids on growth is apparently not related to the ability of medium glucose, ammonium ions, or inorganic phosphate (P^{32}) to pass across the cell membranes, since all of these materials are readily taken up by washed cells in the absence of extracellular amino acids. There is a possibility, however, that added amino acids may have an influence on the intracellular

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Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ont. Part of the program of the Legume Research Committee in Ontario.

accumulation of those intermediates in carbohydrate metabolism which are necessary for the formation of amino acids from ammonia. Such an accumulation might be brought about by directly increasing the rate of carbohydrate degradation or by the prevention, partial or complete, of "oxidative" assimilation. It may be that enzymes bringing about such reactions in the *growing* cell are labile, perhaps adaptive, and are readily degraded in the *resting* cell, requiring preformed amino acids for resynthesis.

The present study was begun in an attempt to elucidate the initiating activity of amino acids on the growth of one particular strain of nodule bacterium and to study the formation of amino acids from ammonium ions, with particular reference to the direct ammoniation of pyruvate.

Methods

Preparation of Washed Cells and Sonic Extracts

Strain R_{21} of *Rhizobium meliloti* was subjected to single-cell isolation by a de Fonbrune micromanipulator and the resulting clones tested for nitrogen-fixing ability (12) on Vernal and de Puit alfalfa and found to be effective. For growth studies 10 ml. amounts of a modified yeast extract – mineral salts medium (17) were inoculated with the test organism and incubated at 30° C. for 24 hours. When necessary, the cells were washed three times in 0.85% sterile saline, using centrifugation, and diluted to a nephelos of 55.0 (0.1 mg. nitrogen/ml.) using a Coleman photonephelometer and a commercially prepared standard. Large quantities of cells were prepared by emulsifying the growth from two 24 hour (30° C.) slant cultures of the organism in 21 ml. of 0.85% sterile saline and adding 10 ml. amounts of this suspension to each of two 2-l. flasks, each containing 1 liter of the yeast extract medium. These flasks were incubated at 27° C. for 24 hours on a reciprocating shaker, the cells removed by centrifugation, washed three times in saline, and brought to a final volume with sterile water or buffer. When sonic extracts were desired the washed cells were suspended in 12 ml. of 0.05 *M* phosphate buffer of pH 8.3 and disruption carried out in a Raytheon 10 kc. sonic oscillator for 5 minutes at a plate current of 1.0 amp. The resulting mixture was then centrifuged for 30 minutes at 3300 $\times$ *g* and the supernatant retained. Micro-Kjeldahl analyses, using a copper–selenium catalyst, revealed an average of 3 mg. of nitrogen/ml. of extract.

Growth and Attempted Adaptation

The ammonium chloride basal medium employed was that previously suggested (13), modified by the omission of sucrose and histidine. The water used in this medium and in all subsequent work was double-distilled from an all-glass apparatus. Sterile, chemically-clean cuvettes were prepared, containing 9.4 ml. of sterile medium and 0.5 ml. of a carbon-source solution (sterilized by sintered-glass filtration), yielding a final concentration of 0.01 *M*. These sources of carbon included arabinose, ribose, xylose, glucose, fructose, mannose, galactose, sucrose, maltose, lactose, sodium lactate, sodium hydrogen malate, sodium pyruvate, sodium succinate, and sodium α-ketoglutarate

and stock solutions were adjusted to pH 7.0 where necessary. The cuvettes were inoculated with 0.1 ml. of *unwashed* cell suspension and incubated at 30° C., together with basal medium controls which were either inoculated or uninoculated, the latter serving as a blank for the daily photonephelometer readings. After the nephelos in each tube had reached at least 50 units the cells were sedimented by centrifugation, washed three times in saline, brought back to the original volume with saline, and 0.1 ml. transferred to the respective cuvettes of a duplicate series. Such cells were termed "adapted". At the same time a similar series of cuvettes was inoculated with 0.1 ml. of a suspension of washed cells obtained from yeast extract – mineral salts medium. These cells were termed "non-adapted".

Growth occurred in the latter two series of cuvettes only on citrate, malate, pyruvate, and α-ketoglutarate and there was little or no reduction in the lag phase by previous growth on the same compounds. Attempts were then made to obtain adapted cells by growing washed cells for several successive transfers in basal medium containing 0.0001 *M* histidine (14), in addition to the specific carbohydrate. No success was obtained, the washed cells not being able to initiate growth in the medium (except with citrate, malate, pyruvate, and α-ketoglutarate) once the histidine was omitted. In addition, no growth occurred when histidine-free carbohydrate medium was supplemented with adenosine mono- or tri-phosphate, or when the initial inoculum was increased up to four times the original amount. Previous studies (9) had shown that vitamins also had no effect on growth initiation of these cells in an amino-acid-free medium.

The effects of increasing the concentrations of certain carbon sources from zero to 0.1 *M*, in 0.01 *M* steps, were studied using unsupplemented basal medium, or basal medium containing 0.0001 *M* final concentration of neutralized L-histidine.

Accumulation of Keto Acids from Glucose

Reaction tubes contained 5 ml. of 0.2 *M* glucose or water, 5 ml. of water and 5 ml. of a suspension (2.6 mg. N/ml.) prepared by adding freshly washed cells to Allison and Hoover's medium (1). In several cases the cells were stored at 5° C. for several days prior to experimentation. A "blank" tube contained Allison and Hoover's medium in place of the cell suspension, while "standard" tubes contained, in place of the cells, 5 ml. (1.6 μM.) pyruvate or α-ketoglutarate solutions. All tubes were incubated at 30° C. and 3 ml. amounts were removed from each reaction mixture and the "blank" at 10 minute intervals from zero time to 30 minutes and placed in the sonorator cup, using 1 ml. of rinse water. Sonation was carried out for 4 minutes and the resulting extract, together with 3 ml. of rinse water, was poured into 3 ml. of freshly prepared 10% trichloroacetic acid and the mixture centrifuged to sediment the protein. The clear supernatant was removed, the sediment washed with 1 ml. of water, and this water added to the original supernatant. Analyses for keto acids were carried out on these fluids according to procedure B of Friedmann and Haugen (8). In some cases, just prior to the addition

of alkali, spectral-transmittance curves were determined for the dinitrophenylhydrazone solutions prepared from the glucose-containing reaction mixtures. Samples of the same solutions were also checked chromatographically against the dinitrophenylhydrazones of known keto acids, using the technique of Cavallini and Frontali (5).

Synthesis of Alanine from Pyruvate

The contents of the reaction tubes are shown in Table I. The diphosphopyridine nucleotide (D.P.N.) added was rapidly reduced by an active

TABLE I

COMPOSITION OF REACTION MIXTURES FOR DEMONSTRATION OF ALANINE FORMATION FROM PYRUVATE AND NH_4^+

Components	Tube No.		
	1	2	3
0.5 ml. 0.05 *M* pH 8.3 phosphate buffer	+	+	+
0.1 ml. 0.5 *M* pyruvate (as the sodium salt)	+	−	+
0.1 ml. 0.5 *M* NH_4^+ (as the chloride)	+	−	−
0.1 ml. 0.013 *M* D.P.N.*	+	+	+
0.8 ml. sonic extract	+	+	+
Double-distilled water	0.4 ml.	0.6 ml.	0.5 ml.

* Diphosphopyridine nucleotide (Nutritional Biochemicals).

Tube 2 is a control to determine the amounts of amino acids formed by proteolysis in the sonic extract.

Tube 3 is a control to determine the total amount of endogenously produced alanine, including that produced proteolytically.

endogenous respiration. The ammoniation reaction was also studied at pH 7.0 and 6.0, with various equimolar concentrations of pyruvate and NH_4Cl, with the D.P.N. omitted or replaced by triphosphopyridine nucleotide (T.P.N.), and with the sonic extract replaced by washed, intact cells. During incubation in a 30° C. water bath, identical samples were withdrawn from all tubes at definite time intervals and placed in a boiling water-bath for exactly 5 minutes, in order to stop the reaction. In some instances acid was used for this purpose, but it was no more efficient than the heating procedure and introduced undesirable ions. The samples were then centrifuged and 0.04 ml. amounts of the clear supernatants spotted on filter paper sheets, together with various concentrations of known amino acids, and chromatographed in water-saturated phenol at 25° C. After development with 0.1% alcoholic ninhydrin the concentrations of amino acids in the mixtures were ascertained by the elution technique of Block (3), followed by spectrophotometric examination of the eluates at 570 mμ. Standard curves were prepared for each analysis by elution of the known concentrations of the acids.

A minute amount of alanine was non-enzymatically produced in solutions containing only chromatographically-pure pyruvate and NH_4Cl, but was virtually eliminated by omitting any heating during the spotting of the chromatograms. The technique was further complicated, however, by the production of free amino acids from the cell-free extracts during the incubation

period, presumably via proteolysis. These acids included aspartate, glutamate, glycine, alanine, valine, and, in some cases, sulphur-containing amino acids as well. In addition, ammonia was produced by amino acid deamination (10, 17). Consequently, there was a considerable "endogenous" production of alanine by transamination of at least three of these amino acids (aspartate, glutamate, and glycine were shown to be amino group donors) with the added pyruvate, as well as by possible ammoniation of the pyruvate by the endogenously-produced ammonia. In order to obtain a reliable estimate of the alanine formed from the *added* ammonium ions the amount of this acid found in tube 3 was subtracted routinely from that detected in tube 1, with the realization that this value would represent a minimum rate of pyruvate ammoniation. Attempts were made to eliminate transamination by the addition of bromosulphalein (2), but, although this compound at a concentration of 1×10^{-3} *M* decreased alanine–glutamic transamination by 69.4% it almost completely inhibited alanine formation from the added ammonium ions. Other inhibitors, such as *p*-phenylenediamine (6), were tried, but with no pronounced success. The synthesis of alanine was compared with the synthesis of certain other amino acids by replacing the pyruvate in the reaction mixtures with other potential NH_4^+-acceptors.

Alanine dehydrogenase was demonstrated in intact cells by spectrophotometric observation at 540 mμ of anaerobic methylene blue reduction. The cells employed were washed twice in double-distilled water, diluted with 0.06 *M* phosphate buffer of pH 7.0, and adjusted to 18% T, at 420 mμ, in the spectrophotometer, using the same buffer as a diluent. This same enzyme was investigated in sonic extracts by spectrophotometrically observing the reduction of D.P.N. at 340 mμ. The reversibility of the reaction was shown in the latter procedure by the addition of ammonium ions (16).

Glucose Oxidation

One hundred milliliters of yeast extract – mineral salts medium were inoculated with the organism and incubated with continuous agitation at 27° C. for 24 hours. The cells were sedimented, washed twice in saline, diluted to 20 ml. with water, and shaken continuously in a 125 ml. flask for 20 hours. The cells were removed by centrifugation, washed twice in Allison and Hoover's medium, and brought up to a volume of 10 ml. with the same fluid. This procedure ensured a very low endogenous respiration. Conventional manometric techniques were used at 30° C.

Results

The data in Table II indicate that growth initiation of the washed cells occurred in an amino-acid-free NH_4Cl medium when the carbon source present was raised to a sufficiently high level. This level varied from compound to compound, but was generally lower with those acids implicated in carbohydrate breakdown. Fig. 1 shows graphically (*a*) the effect of increasing glucose concentration on growth initiation and (*b*) the growth-initiating effect of histidine, which is a very efficient amino acid from this standpoint (14).

TABLE II

MINIMUM CARBON-SOURCE CONCENTRATIONS PERMITTING GROWTH INITIATION IN AMINO-ACID-FREE BASAL MEDIUM CONTAINING NH_4Cl

Sole carbon source	Lowest molarity at which growth is observed	Sole carbon source	Lowest molarity at which growth is observed
Sucrose	0.02	Lactate	0.03
Glucose	0.05	Pyruvate	0.01
Fructose	0.06	Citrate	0.01
Mannose	0.04	α-Ketoglutarate	0.01
Ribose	*	Succinate	0.05
3-Phosphoglycerate	0.01	Fumarate	0.03
Potassium gluconate	0.01	Malate	0.01
Acetate	*		

* Inactive at the concentrations employed (0.01 to 0.10 *M*).

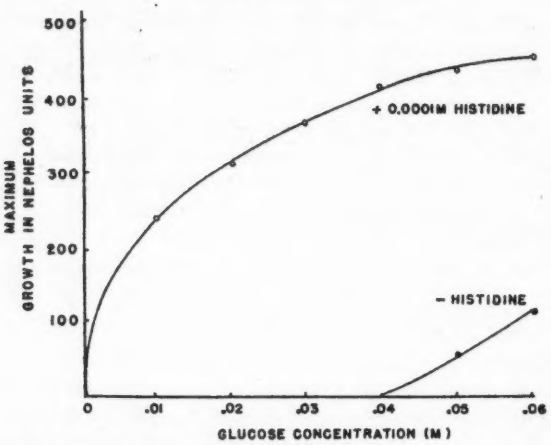
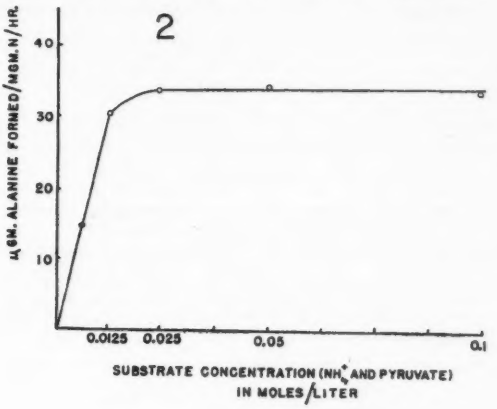


FIG. 1. The growth initiation of washed cells of *Rhizobium meliloti*, strain R_{21}, in media containing increasing concentrations of glucose and in the presence and absence of histidine (0.0001 *M* final concentration). The maximum growth in carbohydrate-free medium containing 0.0001 *M* histidine was 8.0 nephelos units.

Keto acid accumulated linearly when freshly-washed intact cells were incubated in glucose solution; a total of 0.5 μM. of acid being produced in 3 ml. of reaction mixture in 30 minutes. The dinitrophenylhydrazone of this acid had a sharp absorption maximum at 370 mμ, indicating that it was predominately that of pyruvate (15). This was confirmed by chromatographic analysis, which also, however, revealed traces of α-ketoglutarate. The cells stored at 5° C. for several days accumulated no detectable keto acids when examined under similar conditions.

Alanine was synthesized from pyruvate and ammonium ions and of the total alanine detected about 50–60% was formed endogenously and by proteolysis. Sonates proteolytically formed approximately 16 μg. alanine/mg.

cell nitrogen/hour at 30° C. The ammoniation of pyruvate had an optimum pH in the alkaline range, being drastically reduced at pH 7.0 and 6.0, an optimum substrate concentration of 0.025 *M* (Fig. 2), and was stimulated by D.P.N., but not by T.P.N. (Fig. 3). In sonic extracts the amount of alanine synthesized by what appears to be pyruvate ammoniation is similar, on a molar basis, to the amount of glutamate produced by α-ketoglutarate ammoniation (Table III). Oxalacetate and α-ketoisovalerate were inactive as ammonia-acceptors under the conditions employed, while the aspartase-like activity was low. The amount of alanine formed from added ammonium ions by a sonic extract was the same as that found with intact cells, but studies have since indicated (Table IV) that preparations of more than twice



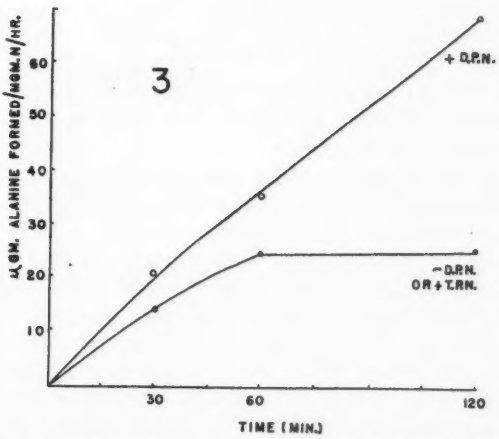


FIG. 2. The effect of substrate concentration on the synthesis of alanine from pyruvate and NH_4^+ by a sonic preparation of *Rhizobium meliloti*.

FIG. 3. The effect of D.P.N. (diphosphopyridine nucleotide) on the synthesis of alanine from pyruvate and NH_4^+ by a sonic preparation of *Rhizobium meliloti*.

this specific activity can be obtained by centrifuging fresh sonic extracts for 48 minutes with a maximum force of 28,000 × *g* (in a Servall vacuum-centrifuge), the upper 3 ml. layer being the most active. This would seem to indicate that the active protein is "soluble" or associated with relatively small particles.

TABLE III

COMPARISON OF VARIOUS ORGANIC ACIDS AS NH_4^+-ACCEPTORS IN A SONIC EXTRACT OF *Rhizobium meliloti*, STRAIN R_{21}

Potential acceptor	μg. amino acid produced from added NH_4^+/hr./mg. cellular N/2 ml. reaction mixture (corrected for endogenous)
Pyruvate	33 (alanine)
Oxalacetate	0 (aspartate)
Fumarate	10 (aspartate)
α-Ketoisovalerate	0 (valine)
α-Ketoglutarate	51 (glutamate)

TABLE IV

THE SYNTHESIS OF ALANINE IN TWO FRACTIONS OF SONIC EXTRACT OF *Rhizobium meliloti*, STRAIN R_{21}

Centrifugation	48 min. at maximum of 28,000 × *g*			
Fraction	Upper 3 ml. layer		Middle 3 ml. layer	
N content/ml. of fraction	2.81 mg.		3.25 mg.	
Alanine formed*	Total 124	Endogenous† 59	Total 102	Endogenous† 77
Alanine formed* from added NH_4^+	65		25	

* In μg./mg. cellular N/hr./2 ml. reaction mixture at 30° C.
† Also includes the alanine produced proteolytically.

Alanine dehydrogenase, potentially responsible for pyruvate ammoniation, was demonstrated in intact cells and sonic extracts of the test organism (Fig. 4) and shown to be reversible.

Washed cells of the test organism oxidized glucose, while the uptake on histidine, α-dinitrophenol, or NH_4^+ alone was meager at the same pH. When histidine and glucose were both present there was a 33.8% stimulation in the oxygen consumption (equivalent to the complete oxidation of an additional 0.25 μM. glucose) at 120 minutes (Fig. 5), after corrections were made for the uptake on histidine alone. This stimulation was not merely an additive effect as a result of the simultaneous oxidation of glucose and histidine, such as Burris and Wilson (4) reported for glucose and glycine, nor was it a result of cellular growth since the response was almost immediate. Under the same conditions, however, identical concentrations of α-dinitrophenol and ammonium ions were, respectively, 8.5 and 5.7 times as active as the histidine in increasing glucose oxidation. These results on the stimulation of glucose oxidation by histidine and ammonium ions are similar to those obtained by other workers (4) using the same species of nodule bacterium.

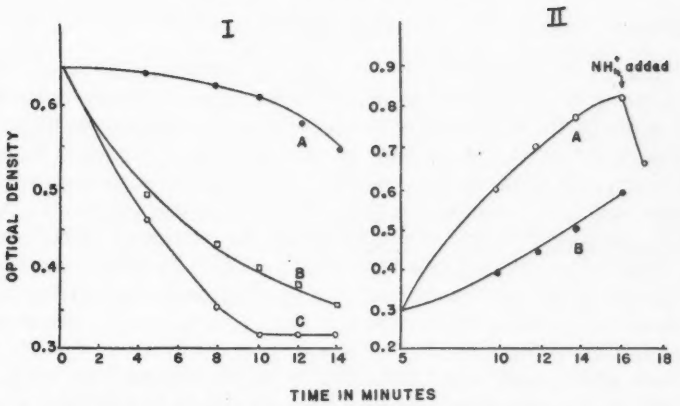


FIG. 4. The presence of alanine dehydrogenase in the test organism.

I. *Intact cells.*—Thunberg tubes contained: 1.5 ml. 1 : 10,000 methylene blue; 4.0 ml. 0.06 *M* phosphate buffer of pH 7.0; 3.0 ml. 0.02 *M* alanine or water; and 0.1 ml. 0.013 *M* D.P.N. or water. At zero time 1.0 ml. of washed-cell suspension was added from the side arm and optical density readings taken at 540 mμ. Curve A, endogenous (−alanine); curve B, + alanine − D.P.N.; curve C, + alanine + D.P.N.

II. *Sonic extract.*—Cuvettes contained: 1.0 ml. 0.05 *M* phosphate buffer of pH 8.3; 1.0 ml. (40 μM.) alanine or water; and 0.5 ml. 0.003 *M* D.P.N. At zero time 0.4 ml. of sonic extract was added and the suspension mixed. After 5 min. optical density readings were taken at 340 mμ. Curve A, +alanine (the arrow signifies the point at which 0.2 ml. 0.7 *M* NH_4Cl was added); curve B, endogenous (−alanine). After the point of NH_4Cl addition curve A is corrected for dilution of the mixture and for the small decrease observed when NH_4Cl was added to the endogenous cuvette.

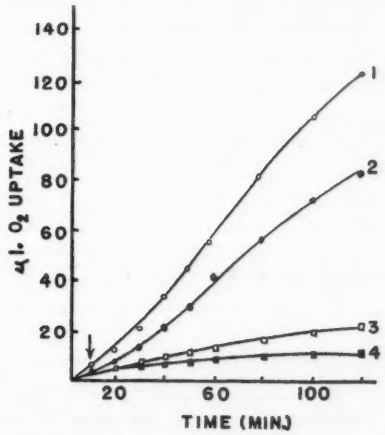


FIG. 5. The oxidation of glucose by washed cells in the presence of histidine. Warburg vessels contained: 1.0 ml. washed cell suspension (in the side arm); 0.9 ml. 0.05 *M* phosphate buffer of pH 8.3; 0.5 ml. of 0.01 *M* glucose or water; 0.14 ml. of water or neutralized L-histidine solution (final concentration: 0.0001 *M*); and water to make a total volume of 2.8 ml. The center well contained 0.2 ml. 20% KOH solution. Curve 1, glucose + histidine; curve 2, glucose − histidine; curve 3, histidine alone; curve 4, endogenous. The arrow signifies the point at which the cells were dumped from the side arm.

Conclusions

It appears that amino acids necessary for growth initiation of the test organism can be supplied (*a*) as intact molecules in the extracellular environment or (*b*) by forcing the cells to synthesize such amino acids by increasing the concentration in the medium of one of those compounds from which NH_4^+-accepting acids can be produced. The necessary concentration levels of these added compounds would be expected to vary, depending on their cellular permeability and their efficiency in producing NH_4^+-acceptors. The present data also indicate that resting cells of *Rhizobium meliloti*, strain R_{21}, possess those enzymes necessary for the degradation of common carbohydrates, such as glucose, and for the formation of amino acids from ammonium ions. In view of this it might be reasoned that the function of trace amounts of intact amino acids is to stimulate, in a low-carbohydrate medium, the accumulation of acceptor acids to a level at which the internal synthesis of amino acids might begin, at which time the process would become "self-initiating". If such is the function of preformed amino acids the accumulation does not appear to take place through an increased oxidative degradation of carbohydrate or a decreased direct assimilation, since ammonium ions and α-dinitrophenol, which do not initiate growth, are more active, respectively, in these phenomena than histidine, which is an excellent growth initiator. The actual function of the growth-initiating amino acid is obscure and it may be that any postulated stimulation such as mentioned above is merely an *indirect* manifestation of the activity of this compound. More logically, perhaps, the initiating acid may be connected with the synthesis of essential protein (probably enzymatic) which has been degraded in the resting cell during washing. This hypothesis is emphasized by the finding (14) that L-amino acid peptides are much superior to their constituent free amino acids as initiating agents, and that such peptides are apparently utilized intact without complete prior hydrolysis, thus suggesting their direct incorporation into protein. The postulated labile protein does not appear to be connected with adaptive enzymes concerned in carbohydrate breakdown, since attempts to demonstrate such enzymes in histidine-grown cells have failed. Although intact cells of *Rhizobium meliloti* are regarded as having weak proteolytic activity in regard to extracellular protein (18), the action against its own protein, as demonstrated in sonic extracts, is appreciable. Evidence that such activity also occurs in intact washed cells of these organisms is emphasized by the low endogenous respiratory quotient of 0.77 and the finding of large quantities of endogenously produced ammonia (10). Therefore, labile enzymatic protein, essential for growth and reproduction, could conceivably be destroyed and require resynthesis from preformed amino acids. At any rate, the activity of the growth-initiating amino acid does not appear to be the result of any simple reaction and any postulations on such activity must remain purely speculative in the absence of detailed information on the modes of carbohydrate degradation and protein synthesis in this organism.

For the synthesis of amino acids from NH_4^+ the test organism employed the activities of L-glutamic acid dehydrogenase and probably that of aspartase. In addition, alanine was formed from pyruvate. The D.P.N. dependency of this latter reaction, coupled with the presence in the cells of a reversible alanine dehydrogenase, indicated that the mechanism involved was probably a direct ammoniation of pyruvate by this enzyme, similar to that noted in sonic extracts of *B. subtilis* by Wiame and Piérard (16) and in intact cells of the same organism by Fairhurst *et al.* (7). The explanation of Kritzman for alanine formation from pyruvate, as summarized by Fairhurst and his coworkers (7), does not apply to the present work since aspartate formation from oxalacetate and ammonia could not be demonstrated in sonic extracts capable of forming alanine.

Acknowledgments

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THE STRUCTURE AND MODE OF DIVISION OF THE NUCLEI IN THE YEAST CELLS AND MYCELIUM OF BLASTOMYCES DERMATITIDIS[1]

A. BAKERSPIGEL

Abstract

The nuclei in the yeast cells and mycelium of *Blastomyces dermatitidis* were studied in the living state and in fixed preparations stained by various techniques. Yeast cells and mycelium are multinucleate. Interdivisional and recently divided nuclei migrate into the yeast buds. Nuclear migration also occurs from one cell to another via the pores in the septa of elongated yeast cells and mycelium. Interdivisional nuclei in yeast cells and mycelium are composed of a spherical Feulgen-negative central body enveloped by granular Feulgen-positive chromatin. A nuclear membrane has not been observed surrounding living or stained nuclei either in yeast cells or mycelium. During nuclear division the chromatin condenses, then elongates, and finally pulls apart to form two sister nuclei. The role played by the central body during nuclear division is obscure. However, it was noted that during the division of the chromatin the central body did not divide but became progressively smaller and apparently disintegrated in the cytoplasm. New central bodies are formed in recently divided nuclei. Mitotic figures such as a spindle or a metaphase plate have not been observed in any living or stained preparations. Complexes of chromosome-like elements were noted in some hyphae, possibly those associated with conidial-bearing stalks. Counts made of such "chromosomes" in several of these complexes revealed a minimum number of six and a maximum of eight.

Introduction

Mycological literature contains very few descriptions of the nuclear cytology of medically important fungi. The most recent contributions of note in this field were those made by O'Hern and Henry (12, 13) on *Coccidioides immitis* and by Drouhet and Zapater (9) on *Paracoccidioides brasiliensis*. A cytological study of yet another of these fungi was made in 1948 by DeLamater (7), who described the structure and mode of division of nuclei in the dimorphic fungus *Blastomyces dermatitidis*. DeLamater (6) also outlined various fixation methods and staining techniques to aid in the study of nuclei in fungi. From his observations he concluded (7) "... it would appear that *Blastomyces* undergoes vegetative nuclear division in a manner comparable to other organisms". However, no other reports either confirming or adding to his observations on the nuclear cytology of *B. dermatitidis* have appeared in the literature.

Cytological studies have been conducted in this laboratory on the nuclei in the *vegetative* spores and mycelium of various fungi (14, 15, 16). These studies have also included a re-investigation of the nuclei in *B. dermatitidis*. The following report embodies the observations made on the structure and mode of division of the nuclei in the yeast cells and mycelium of *B. dermatitidis*.

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Contribution from the Department of Bacteriology and Immunology, University of Western Ontario, Faculty of Medicine, London, Canada. This research was supported by a Professional Training Grant, Provincial Department of Health, Province of Saskatchewan, 1955–57. This work will form part of a thesis to be submitted to the Faculty of Graduate Studies, University of Western Ontario, London, Canada.

Material and Methods

Cultures

Two strains of *B. dermatitidis* were employed during this study. One strain, S-14, was isolated from a human case by Dr. N. F. Conant in 1947. The other strain, S-15, was isolated in 1950 by G. R. Carter from a spontaneous granuloma in the lung of a dog in Montana. Cultures of S-14 and S-15 were sent to the author by Dr. J. W. Carmichael of the University of Alberta. Since the observations made on the nuclei of both strains were similar, only those recorded on S-15 will be discussed in this report.

The yeast phase of *B. dermatitidis* was grown at 37° C. on slants of Sabouraud's dextrose agar medium. The mycelial phase was grown on the same medium at 24° C. Mycelium was also obtained by growing yeast cells on Sabouraud's medium at 24° C. (11).

Fixation

The following fixatives were employed prior to staining nuclei in the yeast cells and mycelium.

1. Acetic acid alcohol.—Prepared by adding 1 part glacial acetic acid to 3 parts 95% alcohol (v/v).

2. Osmium tetroxide.—Prepared as a 2% solution in water and used in the form of vapors.

Stains

1. Giemsa.—Prepared by adding two drops of Giemsa solution (Gurr's Improved Giemsa, R-66) per ml. buffer solution, pH 6.8. This stain has long been used in the study of nuclei in bacteria, blue-green algae, and fungi and its usefulness in these studies has been demonstrated by Robinow (15, 16), Cassel and Hutchinson (5), and Pontefract (14).

2. Heidenhain's iron alum haematoxylin.—Used in the half-oxidized form as outlined by Baker and Jordan (3).

3. The Feulgen test.—The Feulgen reaction to detect the presence of desoxyribonucleic acid (DNA) in the nuclei was carried out in the usual way. "Diamant Fuchsin"* gave brighter positive reactions than other brands of fuchsin.

Cytological Preparations

1. Yeast Cells

Stained preparations of cells in the yeast phase were prepared as follows:

(*a*) *Impression smears.*—A young culture of yeast cells growing on a Sabouraud agar plate at 37° C. was fixed *in situ* by flooding the surface of the culture with acetic acid alcohol. After 10 minutes' fixation, small blocks were cut out and immersed and washed in 70% alcohol for 15 minutes. An agar block was then removed from the alcohol and, after the alcohol was allowed to evaporate, gently impressed, culture side down, upon a clean, grease-free cover slip whose surface had been lightly smeared with 2% egg albumin. The block was then flicked off and the impression smear replaced

* Chroma Gesellschaft Schmidt & Co. Distributed by Roboz Surgical Instrument Co., Washington, D.C.

in 70% alcohol for another 5 minutes. Prior to staining, the smear was hydrolyzed in 1 *N* hydrochloric acid for 12 minutes, followed by washing for a few minutes in distilled water. The smear was then stained in Giemsa for 6 to 9 hours or in Feulgen for 3 hours. After staining in Giemsa the smear was washed in tap water for 3 to 5 minutes and mounted for microscopical examination and photomicrography in either tap water or buffer solution. Following the Feulgen reaction the smear was washed for 60 seconds in SO_2 water, then for 2 minutes in distilled water, and finally for 5 minutes in tap water. To aid in microscopy Feulgen stained preparations were mounted in filtered acetocarmine (10).

(*b*) *Cellophane cultures.*—Yeast cells were also grown on small sterile strips of dialyzing cellophane placed on the surface of a Sabouraud agar plate. Cells to be stained with Giemsa or in Feulgen were fixed *in situ* for 10 minutes with acetic acid alcohol after which the cellophane was stripped from the plate and immersed in 70% alcohol. Hydrolysis in 1 *N* HCl and staining procedures were similar to those described for the impression smears. Yeast cells to be stained with haematoxylin were grown on cellophane and fixed *in situ* by inverting the agar plate over a small glass jar containing 2% OsO_4. The cells were fixed by the osmic vapor for 4 minutes after which the cellophane was stripped from the agar surface and washed in 70% alcohol for at least 1 hour. The cells were then mordanted in 3% iron alum for 2 hours and stained in haematoxylin for 24 hours. The preparation was removed from the stain, washed for 3 minutes in distilled water, and differentiated in 2 to 3% iron alum. Differentiation was watched under the microscope using a water immersion lens. When sufficient differentiation had been obtained the preparation was immersed and washed in two changes of distilled water and then for 10 minutes in two changes of tap water. For microscopical examination such a preparation was dehydrated by passage through a series of 70%, 95%, and absolute alcohols and finally xylene. Permanent mounts were made in D.P.X. synthetic resin.

(*c*) *Preparations for phase contrast microscopy.*—Yeast cells from a young culture were studied with the phase contrast microscope to observe the structure of the living nucleus and its mode of division. Preparations of cells for such studies were made in the following manner. A thin micro-slide was dipped into melted sterile Sabouraud agar and then held up with forceps at one end at an angle to allow excess agar to drain away. After the agar on the slide had hardened it was dipped again into the melted agar and picked up by its opposite end so that an even layer of agar remained on both surfaces of the slide. One surface of the slide was wiped clean, and then with a flattened wire knife agar was removed from the other surface so that only a strip approximately 1 × 1.5 cm. remained in the center of the slide. A few yeast cells from a young culture were then streaked lightly across this strip of agar using a very fine glass fiber. A sterile cover slip was placed over the seeded agar strip and ringed with paraffin leaving one corner open for air. The preparation was examined either soon afterwards or placed for further growth in a moist chamber at 37° C.

2. Mycelium

The mycelial phase was grown at 24° C. on small cellophane strips placed on Sabouraud agar plates. When sufficient growth had occurred preparations were fixed *in situ* and stained in the manner described for the yeast cells. Preparations of mycelium for study with the phase microscope were also made as described for yeast cells. An alternative method employed was to seed small strips of cellophane (1 × 1.5 cm.) placed on Sabouraud medium with yeast cells and incubate these at 24° C. for 4 to 5 days. When sufficient mycelial growth was obtained a cellophane culture was stripped from the agar and immediately placed in a few drops of peptone water or glucose broth on a glass slide. A cover slip was then placed over the preparation, which was sealed with paraffin as described for the yeast cells. This type of preparation was found to be satisfactory for the study of nuclei in the hyphae since the cellophane adhered fairly closely to the under surface of the cover slip.

Microscopy and Photomicrography

1. Light Microscopy

A Zeiss microscope was used which was fitted with a 2 mm. apochromatic objective (N.A. 1.32) in conjunction with a Zeiss achromatic condenser (N.A. 1.4). An interference filter with a transmission peak at 5460 Å and a yellow compensating filter was employed during photography. Koehler illumination was used at all times. Photographs were taken with Kodak Panatomic X cut film.

2. Phase Microscopy

Photographs were taken using a Bausch and Lomb phase contrast condenser and phase objective (N.A. 1.25). Kodak Royal Pan cut film was used for such photography.

Observations

A. The Nuclei in the Yeast Cells

1. The Living Nucleus

It was difficult to keep the same nucleus under continuous observation amidst the crowd of nuclei, mitochondria, and other cytoplasmic inclusions present in a nonbudding cell. Conditions for observation were better in yeast cells which had already budded for in such cells the nuclei were more widely spaced.

As shown in Figs. 1–3, 86, 88*a* interdivisional nuclei in yeast cells are composed of a spherical dense central body surrounded by a less dense cloud-like halo which changes continually from nearly spherical to various angular forms. The nuclei are surrounded by numerous small spherical dense granules in rapid Brownian movement believed to be mitochondria (Figs. 1, 3, 86). At times granular mitochondria may join to form a small beaded chain. Filamentous or rod-shaped mitochondria can also be seen moving to and from the nucleus with a wriggling or undulating motion. At various

times, especially at the beginning of nuclear division, mitochondria may surround the nucleus in such a manner that if one were to examine it at that instant the picture would suggest the presence of a nuclear membrane. However, a true nuclear membrane was not noted surrounding any of the nuclei studied.

Nuclei do not remain static but move about within the cell. Nuclei may move approximately 0.5 to 2.0 μ in any direction within the cell and then swing back to their original position. The first sign that division is to occur is a marked increase in the movement of the nucleus within the cell. The halo surrounding the central body changes its form more often and its intensity fluctuates. The halo then begins to elongate and the central body becomes less dense, smaller, and at times is almost invisible (Fig. 88*c*). Finally the central body may disappear into the cytoplasm and at the same time the halo pulls apart and two small round halos appear side by side (Figs. 88*d*, *e*). The time taken for such a division to occur is usually 4 to 6 minutes. What happens to the central body during division is not entirely clear but it was noted that the dividing halo withdraws from it and that a new central body is formed by each of the sister nuclei. These central bodies can be seen in the two sister nuclei within 30 minutes following division and they first appear as tiny dots which enlarge becoming small round grayish bodies each surrounded by a light halo (Figs. 4; 88*f*, *g*).

The nuclei in the yeast cells do not divide synchronously. It usually takes at least 4 hours before each of the sister nuclei grow to the size of the original nucleus. Growth or maturation of a nucleus was observed to proceed by an enlargement of the central body and its surrounding halo. When these nuclei had reached their maximum size they either remained in this state for a number of hours or divided again in the manner described.

2. Stained Preparations

(*a*) *The Interdivisional Nucleus*

Yeast cells are multinucleate each cell containing at least two nuclei (Figs. 6–8, 11, 12). In the normal-sized and larger (giant) yeast cells nuclei lie clustered (Figs. 6, 7, 11, 12) while in elongated cells the nuclei lie in a straight row parallel to the long axis of the cell (Figs. 13–15). The interdivisional nucleus in HCl–Giemsa and in Feulgen stained preparations is irregularly spherical and granular. In many of these nuclei there may be one or more granules which show a strong Feulgen reaction and stain more heavily with Giemsa (Figs. 6, 11, 12, 14, 75*a–c*, 78*a*, 79*a*). These granules are not apparent in preparations fixed and stained during the final division stages of the nucleus. Though nuclei were more intensely stained with Giemsa than in Feulgen both techniques complemented each other well. In fact no difficulty was encountered in obtaining bright Feulgen preparations revealing the site of DNA in these nuclei (Figs. 11, 12, 14, 15).

Nuclei migrate from the yeast cells into the buds (Fig. 9). These nuclei are granular, elongated, or lens-shaped. Septa may divide an elongated yeast cell into a number of cells each containing at least two nuclei (Figs.

16, 79). These nuclei resemble those in the yeast cells proper. Nuclei also migrate from one cell to another in these elongated yeast forms via the central pores in the septa (Figs. 16, 17, 79*b*).

A nuclear membrane was not noted in any preparations, including those stained with iron alum haematoxylin.

It is interesting to note that nuclei stained with haematoxylin appear similar to living nuclei observed with the phase contrast microscope. In haematoxylin preparations the interdivisional nucleus is composed of a darkly stained central body surrounded by a light gray halo which in Feulgen preparations is the site of the Feulgen-positive chromatin (Figs. 10, 18, 19, 81, 82). It must be emphasized, however, that while in cells of higher plants or in the sexual cells of many fungi the chromatin or chromosomes do stain with haematoxylin, in the vegetative cells of this and other fungi haematoxylin is not a useful nuclear stain. In fact a gray halo can be seen in the haematoxylin stained preparations of *B. dermatitidis* only if differentiation with iron alum is carefully controlled. Similar results with this stain have been obtained in this laboratory with studies of the vegetative nuclei in *Penicillium citrinum* and *P. notatum* (14); *Mucor hiemalis* and *M. fragilis* (15); *Phycomyces blakesleeanus* (16); *Saprolegnia ferax*, *S. parasitica*, *Achlya racemosa*, *Endogone sphagnophila*, and other fungi studied by the author (unpublished data).

(*b*) *The Nucleus during Division*

In HCl–Giemsa stained preparations the chromatin at the beginning of nuclear division becomes condensed and stains more intensely (Figs. 20, 25, 76*a*). Condensation of the chromatin may cause it in many instances to withdraw from and reveal a lighter stained round central body (Figs. 20, 30). At this stage the condensed chromatin may also appear as a densely stained crescent partly enveloping the central body (Figs. 29, 30, 78*b*). As division progresses this crescent becomes pinched and thinner in the middle so that two semilunar or oval shaped bodies are formed which remain attached (Figs. 25; 31; 76*b*, *c*). It now becomes difficult to find the central body, which is usually not evident in preparations fixed and stained at this stage (Figs. 29; 31; 80*a*, *b*). As division proceeds the two condensed chromatin bodies draw further apart and finally separate into small densely stained sister nuclei (Figs. 26, 32).

Feulgen-stained preparations reveal a similar sequence of figures during nuclear division. The Feulgen positive chromatin condenses (Figs. 21–23, 24, 33, 76*d*) and stains more intensely. When such preparations are examined microscopically the early division figures can be picked out with ease since the condensed chromatin is a brighter red than that observed in the interdivisional nucleus. Division proceeds with the chromatin drawing out as shown in the budding yeast cells in Figs. 27, 77*a–d*. In Fig. 27 the dividing nucleus is seen lying in a plane parallel to the long axis of the cell revealing a heavier concentration of Feulgen positive chromatin at either end. Finally the chromatin pulls apart to form two small, round sister nuclei which are intensely Feulgen positive (Figs. 28, 77*e*).

Though the final products of nuclear division are commonly seen in HCl–Giemsa and in Feulgen preparations, the preceding stage, that is the one showing the drawn out chromatin about to separate, is not seen so often. This may be explained on the basis of observations made on living nuclei with the phase contrast microscope. Here the final stage of nuclear division occurred so quickly that it was evident for only a few seconds.

Examination of numerous stained yeast cells has failed to reveal any metaphase plates or spindles.

B. The Nuclei in the Mycelium

1. The Living Nucleus

The best area for the study of a nucleus in a hypha is in a cell situated between the growing hyphal tip and the older vacuolated cells. Though several small and recently divided nuclei can be observed near growing hyphal tips the cytoplasm is so dense in that region that continued observation of nuclei is practically impossible.

As in the yeast cells an interdivisional nucleus in a hyphal cell is composed of a round dense central body surrounded by a light cloud-like halo (Figs. 5, 87, 88*a*). This halo changes its shape continually from round to angular. Small granular mitochondria are also present with filamentous forms most common near hyphal tips and in older and more vacuolated cells. Nuclei move about in the cytoplasm in various directions and they can also be seen migrating from one cell to another via the septal pore. Similar observations had been recorded previously by Dowding and Bakerspigel (8) on the nuclei in the vegetative hyphae of the ascomycete *Gelasinospora tetrasperma.* A nuclear membrane could not be discerned around any of the nuclei studied.

Division of a nucleus in a hyphal cell occurs in a manner similar to that observed in the yeast cells. The nucleus at first becomes very agitated, usually moving backwards and forwards along the wall of the hypha. The halo surrounding the central body may become brighter and stretch in a plane nearly parallel to the long axis of the cell. At times it appears kidney-shaped with the central body situated in the concavity (Figs. 5; 88*b*, *c*). As division proceeds the central body becomes less dense. Finally, with the central body hardly visible, the halo quickly pulls apart into two teardrop-shaped halos (Fig. 88*d*). The whole process of nuclear division usually occurs within 3 to 4 minutes.

As in the yeast cells the fate of the central body of a dividing nucleus in hyphae is obscure. Sister nuclei immediately after division do not appear to contain a central body, yet after a period of rest (usually 30 to 60 minutes) very small intensely dark central bodies are found in the halos (Fig. 88*f*). These small central bodies then enlarge during the maturation of the nucleus (Fig. 88*g*).

From the observations made on nuclei in the yeast cells and hyphae it is postulated that the central bodies do not divide along with the chromatin but become progressively smaller and disintegrate during division of the chromatin. New central bodies are then formed by each of the sister nuclei.

2. Stained Preparations

(*a*) *The Interdivisional Nucleus*

In the mycelium of *B. dermatitidis* interdivisional nuclei stained with HCl–Giemsa appear in two distinct forms. There is an irregularly spherical nucleus (Figs. 34–36, 83*b*) and an elongated racquet-shaped nucleus (Figs. 36–38, 83*c*). Both forms are granular, some of the granules of chromatin being larger than others. It may be that the racquet-shaped nucleus is molded into this shape either by the streaming protoplasm carrying it through a hypha or during its migration into a side branch (Fig. 38). These two forms of the interdivisional nucleus can also be seen in Feulgen stained preparations (Figs. 48–50, 51–55, 83*a*). In addition haematoxylin preparations reveal similar nuclei containing darkly stained central bodies which cannot be seen in Feulgen preparations (Figs. 61–66, 85).

(*b*) *The Nucleus during Division*

Division of a nucleus in a hyphal cell begins with condensation of the chromatin which envelops the central body. In Feulgen and HCl–Giemsa stained preparations the chromatin at this stage appears more compact, granular, and densely stained (Figs. 39, 40, 56, 57, 58, 84*a*). The chromatin not only stains more intensely but because of increased condensation withdraws from and reveals in HCl–Giemsa preparations a large round or oval central body (Figs. 39–43, 83*d*, 84*b*). Condensation may continue till the chromatin is wholly withdrawn from the central body, which is then observed lying free in the cytoplasm (Figs. 42, 43, 44, 84*b*). There is no conclusive evidence as yet whether the central body, which has become progressively smaller, now disintegrates completely, though from this stage of division onward it cannot be observed in HCl–Giemsa stained preparations.

As division proceeds the chromatin begins to pull apart revealing heavier concentrations of chromatin at either end connected by a thin strand (Figs. 45, 46, 59, 84*c*). This strand finally breaks to produce two small round densely stained nuclei of nearly equal size (Figs. 47, 60, 84*d*). A central body cannot be seen in the products of recently divided nuclei.

In some of the hyphae of *B. dermatitidis* there are chromosome-like configurations without any central bodies but containing one or more darker staining granules. These complexes were observed in Feulgen stained (Figs. 71–73) and HCl–Giemsa stained (Figs. 70, 74) preparations but not in haematoxylin stained material nor in hyphae studied with the phase contrast microscope. Similar figures have been observed by Pontefract (14) in *P. citrinum* and *P. notatum*, where they occurred only in the conidiophores and in the hyphae from which the former arose. This is interesting since these penicillia and *B. dermatitidis* are all members of the Fungi Imperfecti. The author has also observed and photographed similar configurations in the conidiophores of another imperfect fungus, *Scopulariopsis brevicaulis*, and in the vegetative hyphae of *Neurospora crassa* and a species of *Phyllosticta* (unpublished data). Whether or not these Feulgen positive complexes are true chromosomes is a matter for further investigation and in

B. dermatitidis a study of them should be made in conjunction with the development of the conidial stage. However, assuming these are chromosomes it would appear that there are a minimum of six and a maximum of eight present in two of these complexes shown in Figs. 71–72 and 89*a*, *b*.

Discussion

Observations made during this study have thrown additional light upon the structure and mode of division of the nuclei in the yeast cells and mycelium of *B. dermatitidis*. In the present study there was good agreement between the behavior of the nuclei in living yeast cells and hyphae, and what could be inferred about them from the study of fixed and stained preparations. However, many of these observations differ from those previously published by DeLamater (7). These differences are due not only to differences in the methods of fixation and staining but also to the interpretation of the recorded observations.

A number of problems arose in attempting to compare present observations with those published by DeLamater (7). One of these was DeLamater's dismissal of the usefulness of the Feulgen reaction in the study of fungal nuclei. This is rather puzzling since in our hands both interdivisional and dividing nuclei were easily stained by this technique and readily comparable with HCl–Giemsa stained nuclei. It is possible that the weakness in DeLamater's Feulgen preparations may have been partially due to fixing cells in Schaudinn's solution at 60° C. for, apparently, 1 hour (6, 7). For example, when yeast cells or mycelium were fixed in this manner a coarse precipitate was produced in the cytoplasm, and nuclei stained with the HCl–Giemsa technique or with basic fuchsin appeared very contracted, "spoked" (Figs. 68, 69), and vastly different to those fixed in acetic acid alcohol, which preserves nuclei well. This fixation effect, which is probably produced by the $HgCl_2$ in Schaudinn's solution, has been discussed by Baker (2) in his book on cytological technique.

Schaudinn's fixative may also have affected the appearance of the stained nuclei in the cells of *Paracoccidioides brasiliensis*, another dimorphic fungus closely related to *B. dermatitidis*. Drouhet and Zapater (9) fixed cells of *P. brasiliensis* in this solution for 2 to 3 hours though no reason was given for such a prolonged and seemingly drastic treatment. Wenrich (17) has reported that fixing the ordinary intestinal protozoa in Schaudinn fluid for 1 minute was just as satisfactory as for a longer period and there was no advantage in raising the temperature above ordinary room temperature. Furthermore their method of first removing the mycelial growth from the surface of the agar medium or the cellophane sheets and *then* putting it into the fixative is open to criticism for just holding mycelium of *B. dermatitidis* (and other fungi) in air for 10 to 15 seconds or longer before fixation produces weirdly shaped nuclei (Fig. 67). In addition these authors studied the stained nuclei of *P. brasiliensis* after dehydrating them in a series of alcohols and acetone. Similar treatment of stained nuclei in *B. dermatitidis*

resulted in their shrinking with loss of stain. Unfortunately most of their drawings and photographs are too small and indistinct to permit critical study of the stained nuclei. A further example of the unfavorable effects of Schaudinn's solution may be seen in the results obtained by Carmichael (4), who also employed DeLamater's technique in fixing and staining nuclei in the conidia, chlamydospores, and vegetative hyphae of the pulmonary fungus *Haplosporangium parvum*.

Difficulty was also encountered in attempting to evaluate DeLamater's interpretation of nuclear structure and mode of division based on the pictorial evidence presented in his publication. Furthermore it is unfortunate that he described the variously shaped nuclei shown in his photographs and drawings in the terminology of classical mitosis. Such descriptions appear inappropriate when one tries to fit them in with present observations made on the nuclei in *B. dermatitidis* and other recent ones made on the nuclei in the vegetative cells of other fungi (14, 15, 16). For example the statement is made that "The next stage observed with assurance is the metaphase". Examination of DeLamater's Figs. 1, L and M, and 3, A to j, shows that an assertion made on the basis of such vague and ambiguous evidence is rather questionable. Another example is the statement, "Following what appears to be a bona fide telephase the chromatinic granules or chromosomes form a dense mass". Upon examination of his photographs *3k* and *3l*, one can see clusters of densely stained nuclei but no clear cut "bona fide telephase" (in the classical mitotic manner) *prior* to the formation of this mass of dense chromatinic granules. In this connection the two densely stained nuclear crescents of the "late telephase" shown in DeLamater's Fig. *4a* could equally well be interpreted as two nuclei in the state of condensation which immediately precedes division.

The scarcity of useful information in the literature on the structure and manner of division of vegetative nuclei of fungi in general has been recently pointed out by Robinow (15, 16) and Pontefract (14). A study of the scanty literature on the cytology of medically important fungi—most of which are only known in the imperfect stage—reveals a similar scarcity to be also present in the few reports on the nuclear cytology of these fungi. However what confuses the reader even more are the statements made by some authors defining nuclear division in the *vegetative* cells of these fungi as being "mitotic". For example, the publication by Baker *et al.* on *Coccidioides immitis* (1), though not a cytological study *per se*, is enlightening in some of the statements made concerning the nuclei in this fungus. The authors complained that the nuclei were too small to be studied and that the cytoplasm retained the nuclear stains as tenaciously as the nuclei. It is possible that the use of other fixation methods and acid hydrolysis before staining might have eliminated this difficulty. However, what is more interesting is their assumption that the nuclei in these vegetative cells divide by the "usual mitosis". Examination of the figures upon which this assumption was based makes one wonder how the interpretation of "usual mitosis" was

reached by the authors. More recently in a paper on the effects of ribonuclease on *C. immitis*, O'Hern and Henry (13) reported that the nuclei in this fungus "were seen to pull apart during division but no achromatic figure was demonstrated by the staining methods used". These authors concede that "while it appears probable that nuclear division takes place by mitosis, further studies will be required to establish this point". Pictorial evidence was not included in their report.

The results obtained during this study suggest that *B. dermatitidis* is another instance of a fungus in which vegetative nuclei do not seem to divide in the classical mitotic manner. Chromosomes are not separately visible in dividing nuclei of *Blastomyces*. The relatively intense Feulgen reaction of *Blastomyces* nuclei about to divide recalls the high chromaticity of chromosomes at mitosis as it is commonly seen in ordinary nuclei. In marked contrast to ordinary nuclei those of *Blastomyces* seem, however, to achieve the segregation of their chromosomes without first lining them up on a metaphase plate and without the aid of spindle fibers.

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EXPLANATION OF FIGURES

PLATE I

FIGS. 1–5. Phase contrast photomicrographs of living nuclei in yeast cells and mycelium.

FIGS. 1–3. Interdivisional nuclei in budding cells. Each nucleus is composed of a large round central body surrounded by an optically less dense angular halo. Granular and filamentous mitochondria can be seen around the nuclei in Figs. 1 and 3.

FIG. 4. Two sister nuclei, the products of a recently divided nucleus, in an elongated bud of a yeast cell. Each nucleus (arrows) is composed of a small round central body surrounded by a light angular halo. This photograph was taken 50 minutes after a nucleus similar to that shown in Fig. 1 had divided.

FIG. 5. A dividing nucleus in a hyphal cell showing the elongated, triangular, or cone-shaped halo situated above the central body. Both ends of this cone-shaped halo (arrows) which are just about to separate appear much brighter than the middle region.

PLATE II

FIGS. 6–12. Stained interdivisional nuclei in cells of the yeast phase.

FIGS. 6–8. *HCl–Giemsa*. Granular spherical nuclei in two nonbudding and one budding yeast cell. Note the coarse granules of chromatin in the nuclei in Fig. 6.

FIG. 9. *HCl–Giemsa*. Two elongated, lens-shaped granular nuclei fixed during their migration from yeast cells into buds (lower right).

FIG. 10. Two nuclei in a young yeast cell stained with iron alum haematoxylin showing the round densely stained central bodies each surrounded by a less dense angular halo.

FIGS. 11 and 12. *Feulgen*. Interdivisional nuclei in a nonbudding and in a budding giant yeast cell. Relatively large Feulgen-positive granules of chromatin are evident in the nuclei in Fig. 11.

PLATE III

FIGS. 13–24. Stained interdivisional, migrating, and dividing nuclei in cells of the yeast phase.

FIG. 13. *HCl–Giemsa*. An unusual number of interdivisional nuclei lying in a row in a unicellular elongated yeast cell.

FIGS. 14 and 15. *Feulgen*. Spherical interdivisional nuclei in elongated yeast cells.

FIGS. 16 and 17. *HCl–Giemsa*. Interdivisional nuclei fixed while migrating from one cell to another via the septal pores in 2-celled elongated yeast cells.

FIGS. 18 and 19. Haematoxylin stained nuclei in elongated yeast cells showing the round, darkly stained central bodies each surrounded by a less dense angular halo. Granular mitochondria are also present in the cytoplasm.

FIG. 20. *HCl–Giemsa*. A nucleus in a yeast cell showing the more condensed chromatin at beginning of division resulting in its withdrawal from the central body (arrow) seen lying free in the cytoplasm.

FIG. 21. *Feulgen*. Nuclei in a giant budding yeast cell. The chromatin at beginning of division has begun to condense and stain more intensely.

FIG. 22. *Feulgen*. Two nuclei in a small yeast cell showing the strongly Feulgen-positive chromatin at early division. Note the intensely stained granule in the upper nucleus giving it the appearance of a "signet ring".

FIG. 23. *Feulgen*. Highly condensed and intensely stained chromatin at beginning of division in an elongated yeast cell.

FIG. 24. *Feulgen*. A dividing nucleus in an elongated yeast cell. The two crescentic chromatin bodies were fixed just after they had become pinched in the middle.

PLATE IV

FIGS. 25–33. Nuclear division in cells of the yeast phase.

FIG. 25. *HCl-Giemsa*. Two nuclei during early division showing the condensed chromatin beginning to pinch in the middle. Note absence of central bodies.

FIG. 26. *HCl–Giemsa*. Recently divided nuclei in yeast cells. In the cell at the lower left the fourth sister nucleus is out of focus.

FIG. 27. *Feulgen*. Three nuclei at end of division in a budding yeast cell. The chromatin of the lower nucleus, which is in focus, is shown pulling apart. Note the heavier concentration of chromatin at either end of this dividing nucleus.

FIG. 28. *Feulgen*. The products of three recently divided nuclei each showing a very strong Feulgen reaction. The sixth sister nucleus is out of focus.

FIGS. 29–31. *HCl-Giemsa.* Nuclei in a budding yeast cell and in two elongated cells showing the more condensed and crescent-shaped chromatin at beginning of nuclear division. In Figs. 29 and 31 the central bodies are barely visible and in the bottom nucleus in Fig. 29 (arrow) the chromatin is seen pinched in the middle prior to pulling apart (compare with Fig. 24).

FIG. 32. *HCl-Giemsa.* Dividing nuclei in an elongated cell. Three nuclei are seen at early division while the fourth at bottom left has already divided.

FIG. 33. *Feulgen.* Two nuclei in an elongated cell showing the highly condensed and intensely stained chromatin at beginning of nuclear division.

PLATE V

FIGS. 34–47. *HCl-Giemsa.* Stained interdivisional and dividing nuclei in mycelium of *B. dermatitidis.*

FIGS. 34–36. Spherical interdivisional nuclei containing "heterochromatic" granules.

FIGS. 35–38. Lens- or racquet-shaped interdivisional nuclei fixed apparently while migrating through hyphae. The bottom nucleus in Fig. 38 is seen migrating into a secondary branch.

FIGS. 39–44. Chromatin in various stages of condensation during nuclear division. In Fig. 44 the chromation of this nucleus is so condensed that the central body can now be seen lying free in the cytoplasm. Note how the central bodies in Figs. 41–44 have become progressively smaller and less densely stained at this stage of division.

FIG. 45. A nucleus showing division progressing with chromatin beginning to pull apart.

FIG. 46. Division just about complete showing the two densely stained sister nuclei still connected by a thin faintly stained strand of chromatin.

FIG. 47. End of division showing the two densely stained sister nuclei after complete separation. Note absence of demonstrable central bodies in the dividing nuclei shown in Figs. 45–47.

PLATE VI

FIGS. 48–60. Feulgen stained interdivisional and dividing nuclei in mycelium.

FIGS. 48–50. Spherical or oval interdivisional nuclei.

FIGS. 51–55. Lens - or racquet-shaped nuclei apparently fixed while migrating through hyphae. The nuclei in Fig. 55 are migrating into a branch (compare with Fig. 38).

FIGS. 56–58. Various stages of condensation of the chromatin at beginning of nuclear division.

FIG. 59. The chromatin of this dividing nucleus is seen pulling apart at end of division. Note the heavier concentration of Feulgen-positive chromatin at either end of the dividing nucleus (compare with Fig. 46).

FIG. 60. Two intensely stained sister nuclei the products of a recently divided nucleus (compare with Fig. 47).

PLATE VII

FIGS. 61–66. Interdivisional nuclei in hyphae stained with iron alum haematoxylin. Each nucleus is composed of a stained central body surrounded by a less dense angular or elongated halo. The elongated nuclei in Figs. 64–66 (arrows) were probably fixed during their migration through the hyphae.

FIG. 67. Nuclei in a hypha fixed in Schaudinn's solution at 60° C. for 3 hours and stained with basic fuchsin. Mycelium was held in air for 10 seconds before fixation.

FIGS. 68 and 69. "Spoked" nuclei in hyphae fixed in Schaudinn's solution at 60° C. for 1 hour and stained with basic fuchsin. Central bodies lacking.

FIGS. 70–74. Chromosome-like configurations in hyphae.

FIG. 70. Stained with HCl-Giemsa.

FIGS. 71–74. Similar figures stained with Feulgen (compare with FIG. 89, *a* and *b*).

PLATE VIII

Semidiagrammatic representation of nuclear structure and mode of division in yeast cells and mycelium of *B. dermatitidis.*

FIG. 75. Three interdivisional nuclei in a yeast cell. The nuclei are roughly spherical and granular and contain one or more granules of "heterochromatin."

a. HCl-Giemsa. b and *c Feulgen.*

FIG. 76. Nuclei in a budding yeast cell at the beginning of division.

a. Condensed chromatin of an HCl-Giemsa stained nucleus revealing a less dense central body.

b and *c.* Further condensation of the chromatin now appearing as intensely stained crescents. In *c* the central body is now faintly visible.

d. Condensed chromatin of a Feulgen stained nucleus. This divisional stage is comparable to the one shown in *b.*

FIG. 77. Nuclear division in a budding yeast cell as seen in Feulgen preparations.
a. The condensed and densely stained chromatin is shown pinched in the middle prior to pulling apart.
b and *c*. Later stage in division showing the sister nuclei pulling apart though still attached by strands of chromatin.
d. End of division showing the two densely stained sister nuclei which have just separated.
e. Postdivision showing the two separated densely stained sister nuclei.

FIG. 78. Nuclei in an elongated yeast cell. Drawn from HCl–Giemsa preparations.
a. Interdivisional nucleus.
b. Nucleus at beginning of division showing the condensed chromatin as a deeply stained crescent around the less dense central body.

FIG. 79. Nuclei in an elongated septate yeast cell.
a. Interdivisional nucleus as seen in Feulgen preparations.
b. An elongated nucleus shown migrating from one cell to another via the septal pore.
c. Early divisional stage showing the chromatin pinched in the middle forming two attached crescents. Compare with Figs. 24 and 29.

FIG. 80. An elongated yeast cell. Two nuclei at beginning of division in an elongated yeast cell. Drawn from Feulgen stained preparations. Compare with nuclei drawn in Fig. 77.

FIG. 81. Interdivisional nuclei in a yeast cell as seen in iron alum haematoxylin preparations. Each nucleus is composed of a round darkly stained central body surrounded by a less dense angular halo. In addition granular and filamentous mitochondria are also present in the cell.

FIG. 82. Nuclei in a septate elongated yeast cell as seen in iron alum haematoxylin preparations. Compare with those seen in Fig. 81.

FIG. 83. Nuclei in a hyphal cell.
a. Interdivisional nucleus seen in Feulgen preparations.
b and *c*. Interdivisional nuclei seen in HCl–Giemsa preparations. Granules of various sizes are present in the nuclei shown in *a*–*c*.
d. Nucleus at beginning of division as seen in HCl–Giemsa preparations. The chromatin is more condensed and has withdrawn from the less densely stained central body.

FIG. 84. Nuclear division in a hyphal cell as seen in HCl–Giemsa preparations.
a. Chromatin shown condensed during early division and beginning to withdraw from the central body.
b. Further condensation of the chromatin. The central body is now lying free in the cytoplasm.
c. End of division showing the densely stained sister nuclei still attached by a thin strand of chromatin.
d. Postdivision showing the two separated densely stained sister nuclei. Central bodies are not evident in *c* or *d*.

FIG. 85. Interdivisional nuclei in a hyphal cell stained with iron alum haematoxylin. Each nucleus is composed of a densely stained central body surrounded by a less dense halo. The elongated cone-shaped nucleus at the left depicts a migrating nucleus. Granular and filamentous mitochondria are also present in the cell.

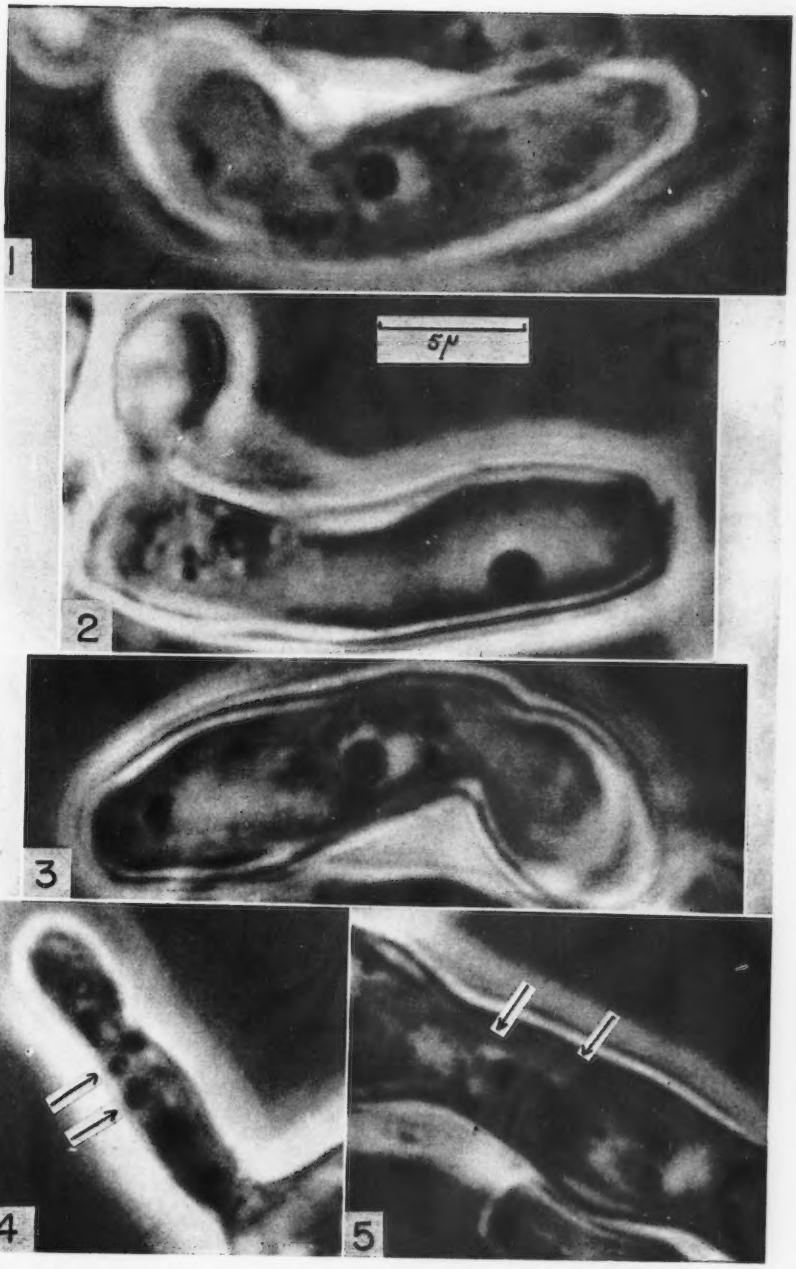
FIGS. 86 and 87. Living interdivisional nuclei and mitochondria in a budding yeast and hyphal cell, respectively. Each nucleus is composed of a round optically dense central body surrounded by a less optically dense round, oval, or angular halo.

FIG. 88. Composite drawing describing the division of living nuclei in yeast and hyphal cells as seen with the phase contrast microscope.
a. A representative interdivisional nucleus.
b. An interdivisional nucleus showing the halo changing in shape from the angular form seen in *a* to one with a tail or beak.
c. Beginning of division showing the halo much more elongated and narrower in the middle situated to one side of the central body, which now appears less dense.
d. Division continuing showing the elongated halo just pulled apart from two wedge-shaped halos. The central body is now smaller than that in *c* and also less dense.
e. Two small tear-shaped halos observed after division. Note absence of central bodies in these halos. Time taken for complete division as shown (from *c*–*e*) approximately 4 minutes.
f. Approximately 30 minutes after division tiny dot-like central bodies appear within the halos.
g. Maturing nuclei showing that both the halos and central bodies have increased in size.

FIG. 89. Drawings of the chromosome-like elements observed in some hyphal cells.
a. Compare with configuration in Fig. 72.
b. Compare with configuration in Fig. 71.

NOTE: Figs. 1–89 follow.

PLATE I



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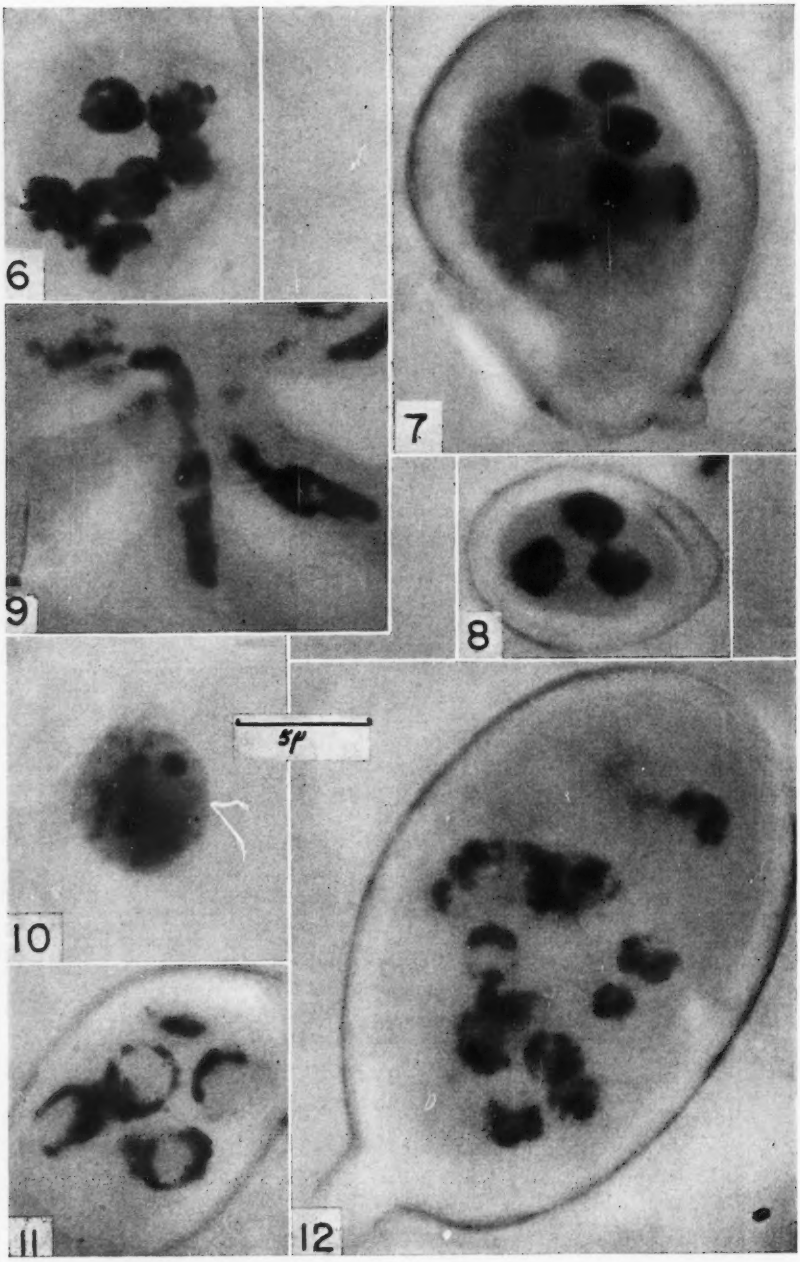

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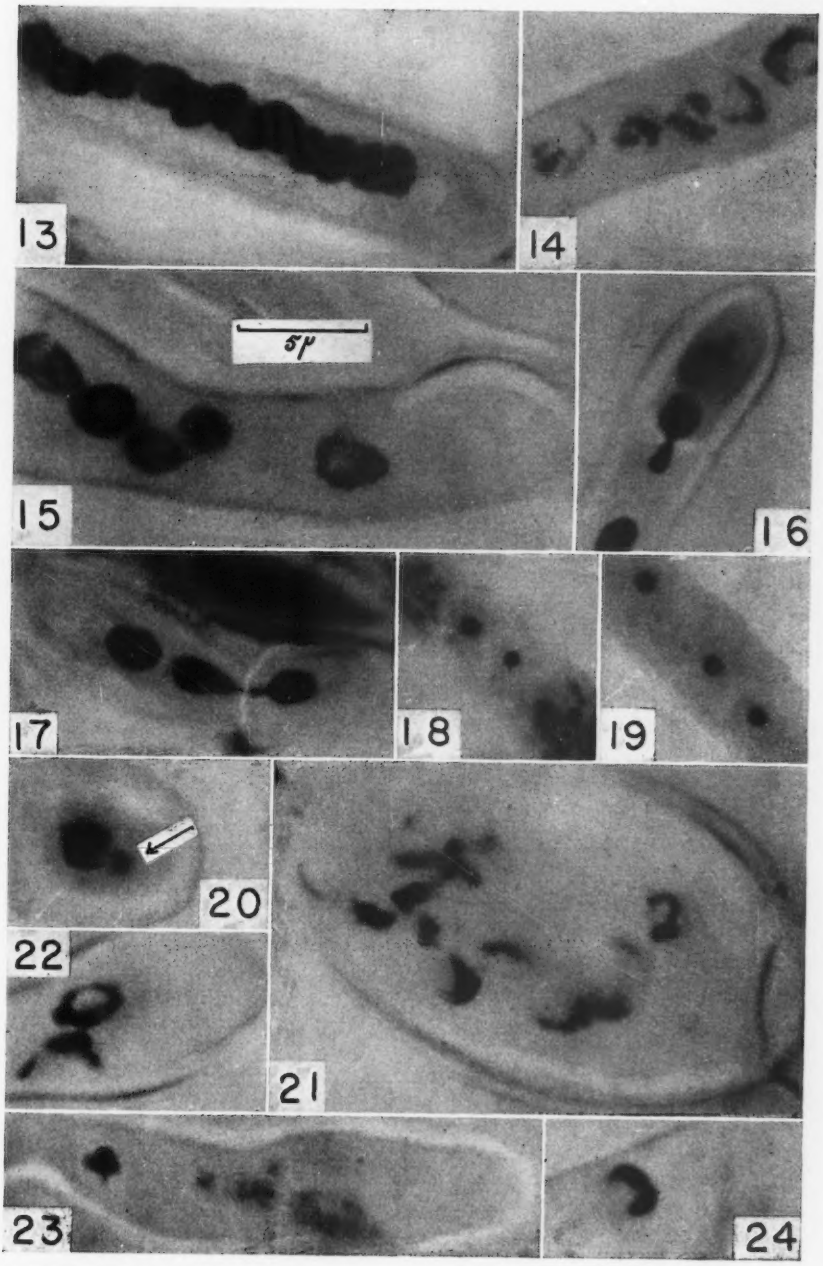

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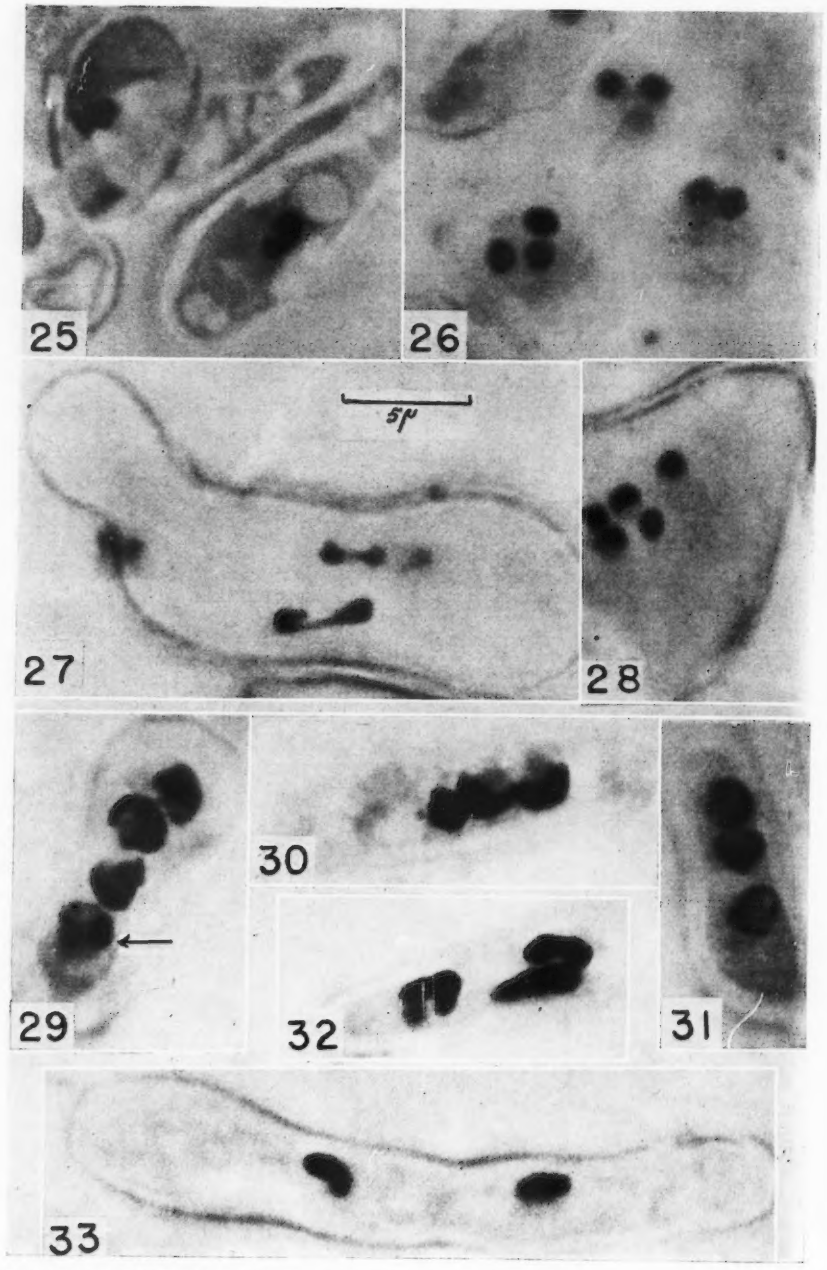
PLATE IV



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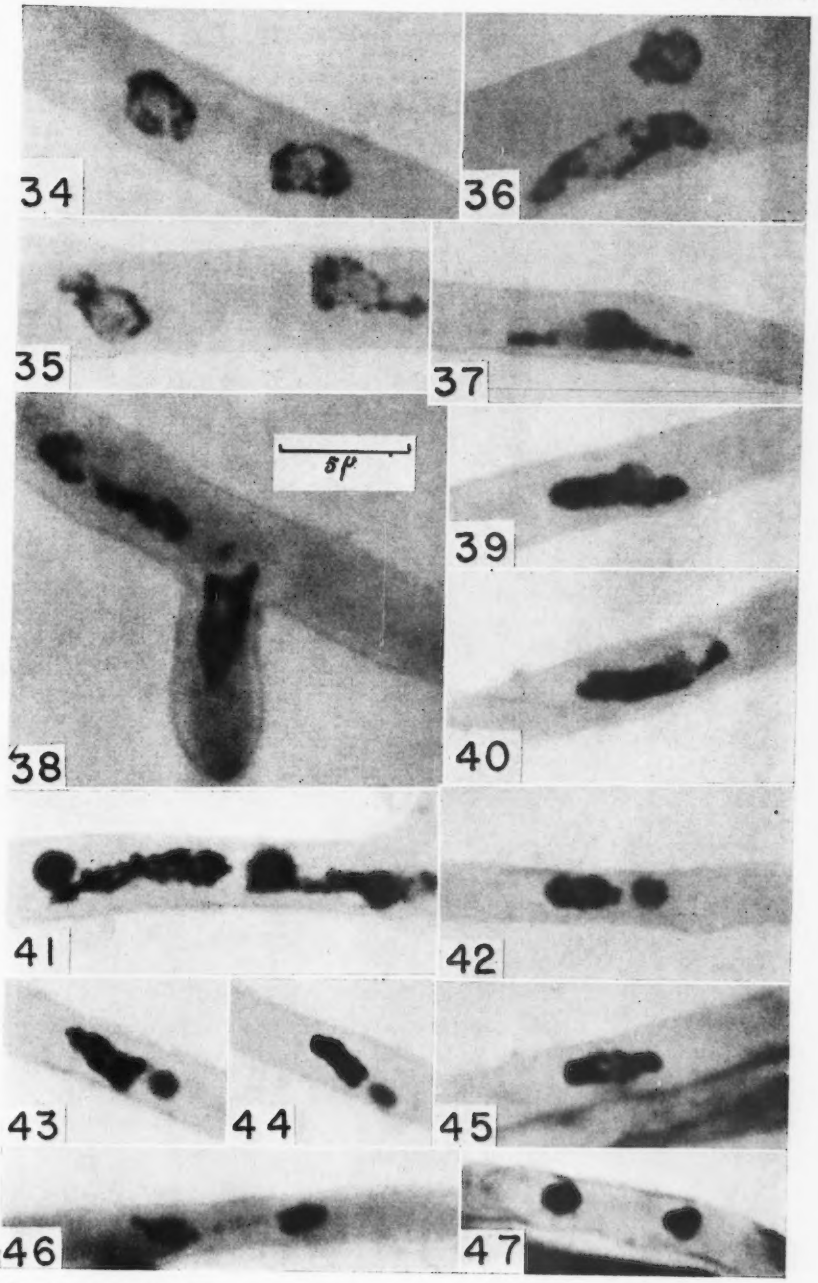

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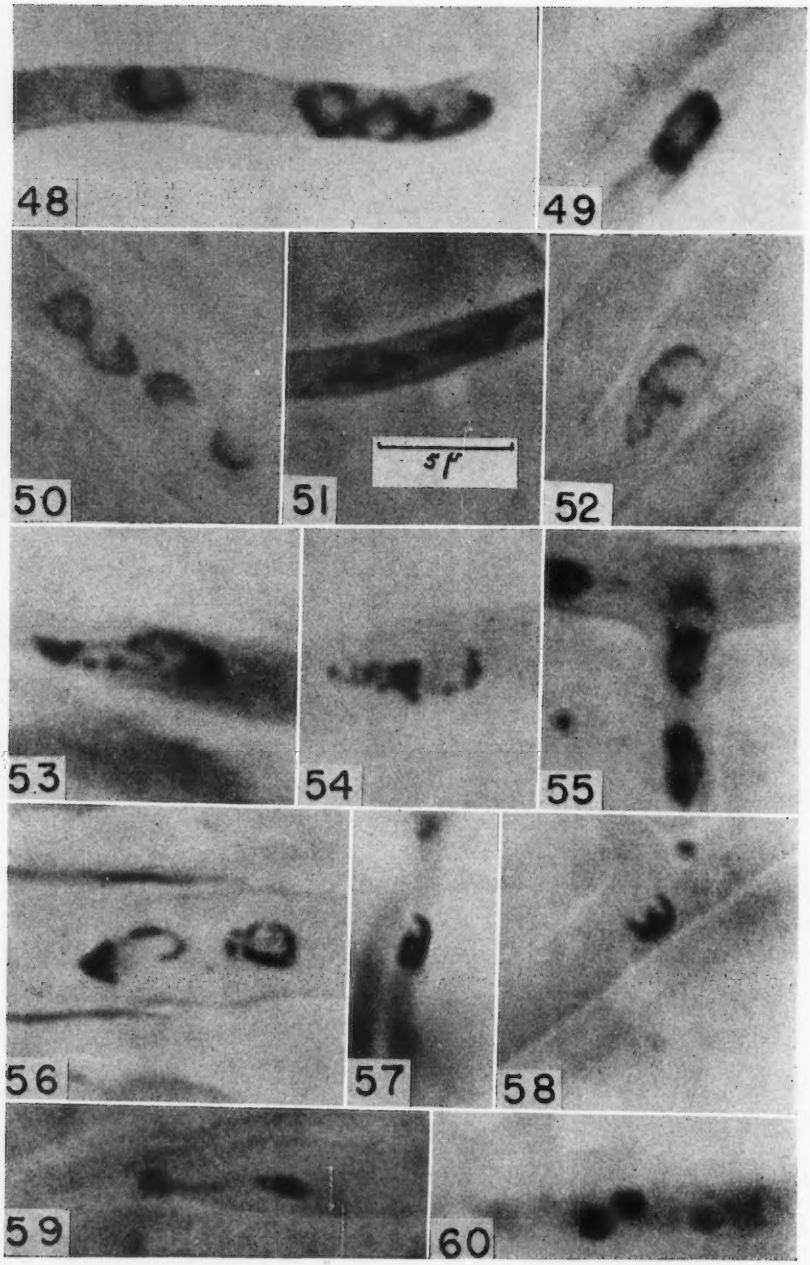

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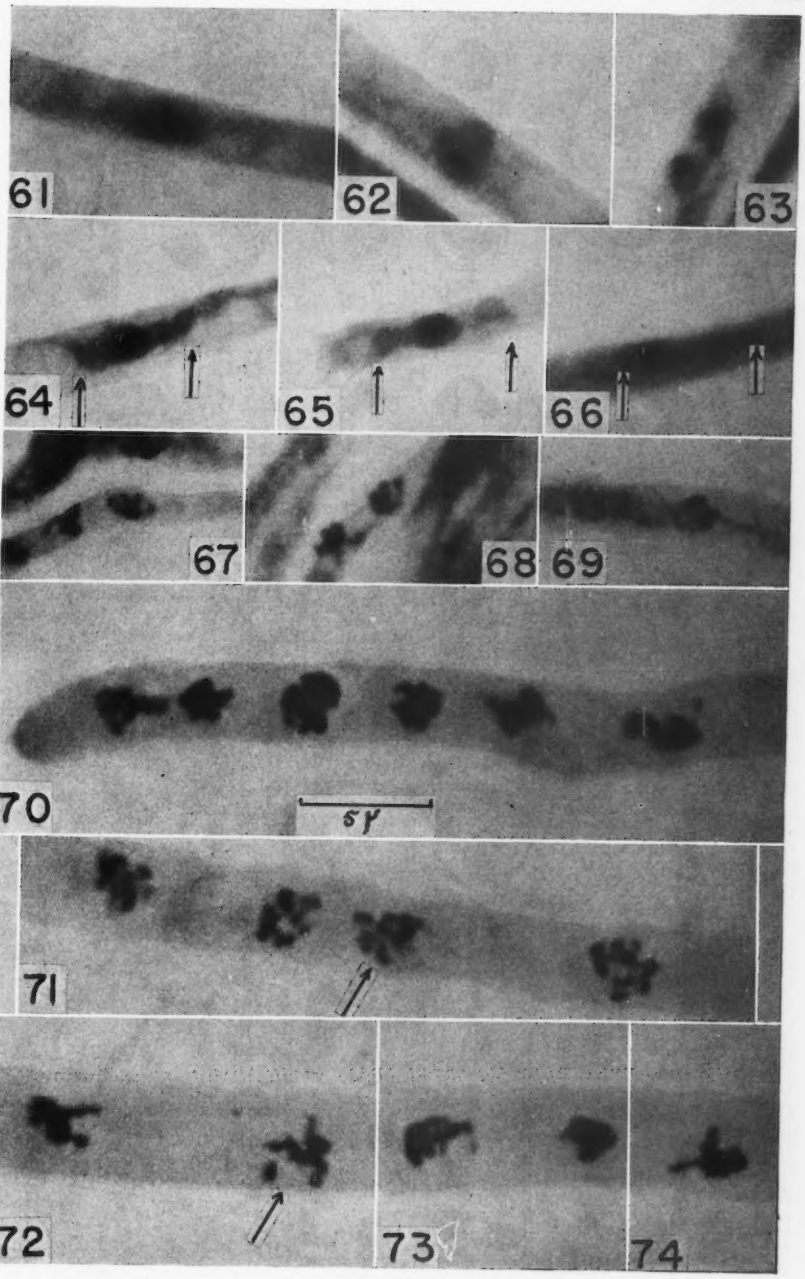

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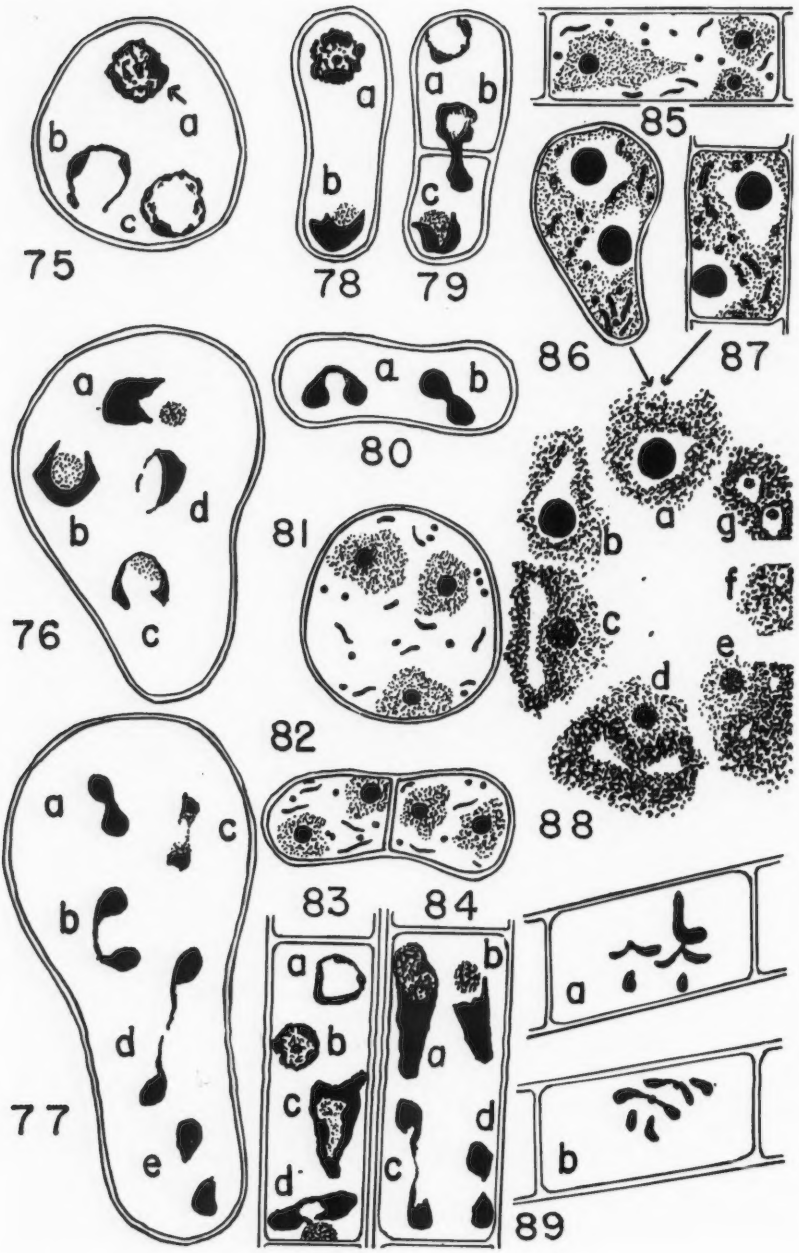

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Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½ × 11 in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered.

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(ii) ***Abstract***

An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

(iii) ***References***

References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should be given and inclusive page numbers are required. The names of periodicals should be abbreviated in the form given in the most recent *List of Periodicals Abstracted by Chemical Abstracts.* All citations should be checked with the original articles, and each one referred to in the text by the key number.

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